

2015

Cellular, molecular, and evolutionary mechanisms of Wolbachia stem cell niche tropism in *Drosophila*

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BOSTON UNIVERSITY
GRADUATE SCHOOL OF ARTS AND SCIENCES

Dissertation

**CELLULAR, MOLECULAR, AND EVOLUTIONARY MECHANISMS OF
WOLBACHIA STEM CELL NICHE TROPISM IN *DROSOPHILA***

by

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B.S., Bridgewater State College, 2008

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

2015

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ACKNOWLEDGMENTS

I would first like to thank Horacio Frydman. I'm truly grateful to have had you as my mentor. You've shown me how to be a rigorous scientist, while still having passion and enthusiasm for my work. I'm so grateful for the opportunities you've provided for me. I entered the program as a master's student and wanted to do research, but I had never done research before. Once I realized how much I enjoyed doing research, my project, and the lab, he fully supported me joining the PhD program. We've gone to numerous conferences, where I've gotten to visit places I otherwise probably would never have gone. And thank you for just your support in general, both in Science and in life. I know I can be difficult sometimes, but I'm very grateful for your mentorship, and your friendship.

I would also like to recognize and thank other BU faculty members for their support and advice throughout my PhD career. My committee members (Kim McCall, Tom Gilmore, Geof Cooper, and Ulla Hansen) have been especially helpful over the years, as well as other faculty who have consulted on some of the evolutionary biology aspects of my project (Mike Sorenson, Chris Schneider, and Sean Mullen). In addition to the faculty, the administrative support staff have been incredibly helpful. I would like to thank Todd Blute for microscopy help. I am grateful to Dennis Batista and Peter Castellano for keeping LSEB up and running and always helping me with little things like booking rooms for lab meeting and printing posters last minute. Meredith Canode has been incredibly helpful with all of the paperwork involved in graduating and making sure everything was in on time, but has also become a friend to me and many other graduate students.

The *Drosophila* and *Wolbachia* communities have also been incredibly helpful and supportive of my thesis work. I would not have been able to analyze many of the *Drosophila* species or *Wolbachia* strains had they not been so willing to share their fly stocks and reagents. A list of researchers includes, but is not limited to: Bill Sullivan, Kostas Bourtzis, Jack Werren, Michael Clark, John Jaenike, Virginie Orgogoza, David Stern, Ruth Lehmann, Kim McCall, and Norbert Perrimon.

I would like to also thank the members of the Frydman lab, both past and present for their support and encouragement. Eva Fast was my scientific partner in crime and lab bay buddy, as we were the first two graduate students in Horacio's lab. Thank you for your friendship. You have also taught me so much about how to be a good scientist, always being critical of your own data, and being determined! The three graduate students who joined the lab following me, Mark Deehan, Rama Krishna Simhadri, and Ajit Kamath, have really been there for me both as fellow scientists, and as friends. Thank you for always challenging me scientifically and always being willing to bash our heads together to figure out why experiments weren't working, or interpreting results that didn't make sense. I'd like to especially thank Ajit for continuing the research involving Armadillo modulating *Wolbachia* levels in the niche. This project was 'my baby' and the first idea that I had conceived on my own. I'm sad to leave the project behind, but I'm glad that I'm leaving it in very capable hands! I'd also like to thank the two newest members of the Frydman lab, Michaela Smith and Luis Ortiz. Although I haven't worked with them for long, they both bring new unique perspectives and exciting ideas to the lab and I'm sure they are going to do excellent work! I'd also like to thank Kanchana Panaram who was a postdoc when I

joined the lab. She began the tropism project in the females and set up a really great story for me to finish. She also trained me in a lot of the techniques I used in my research.

I'd also like to thank all of my fellow graduate students in my program, especially my class-mates, Ben Carr, Kellie Cotter, Brett DiBenedictus, Iker Etchegaray, Sarah Sullivan, Allison Timmons, and Guan Wang. I'm glad that we've all become friends and could not have made it through grad school without our fun nights out and lunch breaks on horrible science days!

I'd finally like to thank my friends and family for their unwavering support throughout my education, but especially in the last 6 years. My best friend, Belinda Smith, has been by my side for 20+ years. Thank you for being a shoulder to lean on, an ear to listen, and for keeping me sane when I wasn't sure if I was going to survive. I'd like to thank my mom, Julie Toomey, for always being there to listen and offer advice, even when she didn't understand the science, she somehow knew exactly what to say. You've taught me strength and determination and always encouraged me to persevere. I'd also like to thank the rest of my immediate family (Dad, Matt, and Jen), my extended family, and my in-laws, for always being there for me and supporting me through this journey. Finally, there are no words to explain how much I'd like to thank my husband, Matt. Thank you for your unconditional support and encouragement. Even if you didn't really want to know, and you knew I would babble on and on about it, you always asked how my experiments were going and how my day went. I truly would not have made it this far without you there supporting me and encouraging me every day.

**CELLULAR, MOLECULAR, AND EVOLUTIONARY MECHANISMS OF
WOLBACHIA STEM CELL NICHE TROPISM IN *DROSOPHILA***

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ABSTRACT

The intracellular bacteria *Wolbachia* infect up to 40% of all insect species, including the vectors of prevalent infectious diseases such as Dengue and malaria. Even though *Wolbachia* infections are the largest pandemic on this planet, the cellular and molecular mechanisms for bacterial spreading in nature are still unknown. *Wolbachia* are mainly vertically transmitted through the egg cytoplasm, however there is also evidence of extensive horizontal transmission. We have found that *Wolbachia* target the stem cell niches in the *Drosophila* ovary to enhance germline colonization and subsequent vertical transmission. This tropism is pervasive across the *Drosophila* genus, with the pattern of targeting being evolutionarily conserved. Phylogenetic analyses, confirmed by hybrid introgression and transinfection experiments, demonstrate that bacterial factors are the major determinants of differential patterns of niche tropism. Furthermore, bacterial load is increased in germline cells passing through infected niches, supporting previous findings suggesting a contribution of *Wolbachia* from stem cell niches towards vertical transmission.

If niche tropism is important for *Wolbachia* transmission through the germline, evolutionary theory predicts that there should be no selective pressure to maintain niche tropism in males. Indeed, we have found that tropism to the stem cell niche in the testis, known as the hub, is not evolutionarily conserved. Towards identifying the cellular and molecular mechanisms of stem cell niche tropism, we investigated hub targeting of closely related *Wolbachia* strains (*wMel*-like strains: *wMel*, *wMel2*, and *wMel3*; *wMelCS*-like strains: *wMelCS*, *wMelCS2*, and *wMelPop*). *wMel*-like and *wMelCS*-like *Wolbachia* strains differ in their frequencies and densities of hub infection. The targeting differences of these strains of *Wolbachia* indicate that this phenotype is rapidly evolving, as they shared a common ancestor only 8,000 years ago. With the plethora of tools available in *D. melanogaster*, a candidate gene approach was used to target host proteins enriched in the stem cell niche in the testis for RNAi mediated gene knockdown in the hub. We have identified *Drosophila* stem cell related signaling pathways that promote *Wolbachia* accumulation. Unraveling the cellular and molecular bases of tissue tropism is fundamental to understanding *Wolbachia*-host interactions.

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LIST OF ABBREVIATIONS

3D	three dimensional
7d	7 day
AJ	adherens junction
Ae.	Aedes
Ank	ankyrin
Arm	Armadillo
Bam	bag of marbles
BMP	bone morphogenic protein
BR	border region
BSA	bovine serum albumin
C	Celsius
CC	cap cells
CI	cytoplasmic incompatibility
CO ₂	carbon dioxide
CySC	cyst stem cell
DEC	<i>Drosophila</i> E-Cadherin
DEPC	diethylpyrocarbonate
DNA	Deoxyribonucleic acid
Dpp	Decapentaplegic
DSHB	Developmental Studies Hybridoma Bank
EtOH	ethanol
FC	follicle cell
Gbb	glass bottom boat
GOI	gene of interest
GSC	germline stem cell
GSCD	germline stem cell division
GSCN	germline stem cell niche
H ₂ O ₂	hydrogen peroxide
HF	high frequency
IS5	insertion sequence 5
ISH	<i>in situ</i> hybridization
JAK-STAT	janus kinase-signal transducer and activator of transcription
L3	third larval instar stage
LF	low frequency
MF	moderate frequency
miRNA	microRNA

mod ⁺	modified
NE	newly eclosed
NGS	normal goat serum
PBANG	phosphate buffered saline with Triton-X 100, bovine serum albumin and normal goat serum
PBS	phosphate buffered saline
PBS/BSA	phosphate buffered saline with bovine serum albumin
PBT	phosphate buffered saline with Triton-X 100
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC	primordial germ cell
PC	polar cell
qPCR	quantitative PCR
resc ⁺	rescue factor
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
ROS	reactive oxygen species
RT	room temperature
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
SSC	saline-sodium citrate
SSC	somatic stem cell
SSCN	somatic stem cell niche
Tet	tetracycline
TF	terminal filament
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UAS	upstream activation sequence
Upd	Unpaired
VNTR	variable number tandem repeat
W-	<i>Wolbachia</i> uninfected <i>Drosophila</i>
W+	<i>Wolbachia</i> infected <i>Drosophila</i>
Wnt	int/Wingless

CHAPTER 1 Introduction

1.1 *Drosophila* as a model organism

For over 100 years, *Drosophila melanogaster* has been used as a model organism to study a diverse range of fields, ranging from neurobiology to evolutionary biology (Ejsmont *et al.*, 2014). The variety of genetic tools, short generation time, and ease of transformation contribute to the wide use of this model system. The approximately 200 Mb genome was the second animal genome to be sequenced (the first by shotgun sequencing), with the first assembly being completed in March of 2000 (Adams *et al.*, 2000; Bosco *et al.*, 2007). With the sequencing of the genomes of several other *Drosophila* species (Ashburner, 2007), comparative genomics across the genus provides a platform for the analysis of coding and non-coding DNA. This makes *Drosophila* an excellent model for evolutionary biologists. Also, the conserved developmental strategies allows for the broad application for developmental biology and human diseases. Notably, *Drosophila* has been extensively used to study signaling pathways, with many of the major components of well-conserved pathways being discovered in this model, including components of the Wnt, Notch, Hippo, Hedgehog, and Planar Cell Polarity pathways (Ejsmont *et al.*, 2014). These high impact discoveries can be attributed to many of the genetic tools that have been developed in *Drosophila*. A useful tool is the GAL4-UAS system (described in detail in Methods, section 2.1.1), which allows for tight spatio-temporal regulation of the expression of a given gene, making *Drosophila* an excellent model system to study a wide range of topics (Brand *et al.*, 1993).

1.1.1 Stem cell niches and stem cells in the ovary

A *Drosophila* female has two ovaries, each comprised of several strings of developing egg chambers, called ovarioles (Figure 1.1). At the most anterior tip of each ovariole is the germarium, a structure which harbors all of the cells necessary to make an egg (Figure 1.2). The germline stem cell (GSC, Figure 1.2, yellow) asymmetrically divides with one daughter progeny eventually producing the oocyte, and the other self-renews and remains a germline stem cell. The daughter progeny then undergoes four rounds of incomplete cytokinesis, becoming a cyst of germline cells interconnected via ring canals. The somatic stem cell (SSC, also called the follicle stem cell, Figure 1.2, light blue), located at the border between regions 2a and 2b of the germarium, also asymmetrically divides to produce a transiently amplifying daughter cell with the other daughter cell self-renewing to remain the SSC. The transiently amplifying follicle cells envelope the germline cysts as they move posteriorly through the germarium. Germline cysts develop into egg chambers as they exit the germarium, and progress through 14 stages of oogenesis, culminating in the mature egg (Spradling, 1993; Kirilly *et al.*, 2007).

Each stem cell population resides in a specialized microenvironment, referred to as the stem cell niche. The stem cell niche provides factors that promote self-renewal to retain the stem cell fate. The germline stem cell niche (GSCN) is comprised of the cap cells (CC, Figure 1.2, dark green) and the terminal filament cells (TF, Figure 1.2, light green). There are 3-5 cap cells located at the base of the germarium in direct contact with the GSCs anchoring them via adherens junctions. The 8-10 disc-like TF cells, although not directly contacting the GSCs, also play a role in GSC maintenance. The escort cells (Figure 1.2,

gray), a stable non-dividing population of cells attached to the basement membrane of the germarium, support the progression of early germline cysts in region 1 and 2A of the germarium, and have also been reported to be important for GSC function (Decotto *et al.*, 2005). The somatic stem cell niche (SSCN) is more ambiguous, with reports indicating that the most posterior escort cell acts at the SSCN providing factors necessary for SSC self-renewal (Sahai-Hernandez *et al.*, 2013).

1.1.2 Stem cell niches and stem cells in the testis

The *Drosophila* testis is an elongated tube that is closed at the end and connected to the rest of the genital tract at the basal end (Figure 1.3B). At the apical tip of each *Drosophila* testis is a group of 10-15 somatically derived cells that form the stem cell niche, called the hub (Figure 1.3C, green). Surrounding the hub are 6-9 GSCs (Figure 1.3C, yellow), which are anchored to the hub via adherens junctions. Each GSC is flanked by two cyst progenitor cells (CySCs, Figure 1.3C, blue). The CySCs are also in contact with the hub through thin cytoplasmic extensions between the GSCs, with their nuclei located further from the hub than the GSCs (Hardy *et al.*, 1979).

1.1.2.1 Intrinsic and extrinsic regulation of stem cell division in the testes

As in the ovary, when a GSC divides in the testis, it does so asymmetrically with one daughter cell remaining in contact with the niche, retaining the stem cell fate, and the other being displaced from the niche and differentiating. The asymmetrical division of GSCs in the testis results from the orientation of the centrosomes and spindles beginning during interphase continuing through mitosis (Yamashita *et al.*, 2005; Pereira *et al.*, 2011). The mechanism controlling centrosome orientation is intracellular, depending upon

polarity cues from the GSC-hub interface (Yamashita *et al.*, 2003). The asymmetrical division of the CySCs is different from the GSCs, with the orientation of the centrosomes being random and repositioning to the hub-CySC interface during anaphase (Cheng *et al.*, 2011).

The maintenance of stem cells in the testis is complex, as two distinct stem cell populations reside in the same niche. In complex stem cell environments, such as this, it is common that one stem cell type receives and responds to signals from the other, as well as from the niche. This occurs in the hub:GSC: CySC system, where the CySCs (in addition to the hub) provide cues important for GSC self-renewal (Leatherman *et al.*, 2008). Figure 1.4 summarizes several signaling pathways active in the hub.

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway was the first pathway discovered to modulate stem cell activity in the *Drosophila* testis. An interleukin-like cytokine, Unpaired (Upd), is secreted by the hub and activates the Janus kinase (JAK) signaling pathway in both GSCs and CySCs by binding to its receptor, Domeless. The Upd ligand is glycosylated and is tightly associated with the extracellular matrix after secretion, limiting its diffusion (Harrison *et al.*, 1998). The activated downstream effector, Signal transducer and activator of transcription (STAT) translocates into the nucleus and activates transcription of Stat responsive genes (Figure 1.4) (Hombria *et al.*, 2002). JAK-STAT signaling is required in the stem cells for their maintenance, as depletion of STAT results in loss of stem cells (Kiger *et al.*, 2001; Tulina *et al.*, 2001). Furthermore, STAT activation in just the CySCs is sufficient for GSC self-renewal, demonstrating that signals from the CySCs in addition to those from the hub are

important for GSC maintenance (Leatherman *et al.*, 2008). In CySCs, JAK-STAT signaling is necessary and sufficient for self-renewal. Downstream targets of this pathway, the zinc-finger transcription repressor, *zfh1* (*zinc-finger homeodomain 1*) and the nuclear factor *chinmo* (*chronologically inappropriate morphogenesis*), were identified in CySCs as necessary to maintain their undifferentiated status (Leatherman *et al.*, 2008; Flaherty *et al.*, 2010).

Bone morphogenetic protein (BMP) signaling is also important for the maintenance of stem cells in the testes. BMP ligands Decapentaplegic (Dpp) and Glass bottom boat (Gbb) are expressed by the hub and CySCs and activate signaling in the GSCs. This signaling is required for the transcriptional repression of the differentiation signal *bag of marbles* (*bam*), which is expressed in the daughter cells promoting differentiation (Chen *et al.*, 2003; Shivdasani *et al.*, 2003; Kawase *et al.*, 2004; Schulz *et al.*, 2004).

In addition to signaling pathways, cell-cell adhesion between stem cells and the niche anchors stem cells within the niche and close to self-renewal signals. *Drosophila* E-Cadherin (DEC) is required for the maintenance of both GSCs and CySCs, through anchorage to the hub. In addition, DEC is a component of adherens junctions, which with α - and β -catenin (Armadillo), is thought to be important for transducing BMP signaling from the hub to the stem cells (Michel *et al.*, 2011).

1.2 *Wolbachia*, obligatory intracellular endosymbiont

Wolbachia are obligatory intracellular alphaproteobacteria of the order Rickettsiales. Unlike related genera (*Anaplasma*, *Ehrlichia*, *Rickettsia*) that can be found in both invertebrate and mammalian hosts, *Wolbachia* exclusively infect invertebrate hosts.

Wolbachia's association with their invertebrate hosts can range from pathogenic (i.e., *wMelPop*'s severe life-shortening phenotype (Min *et al.*, 1997)) to mutualistic (i.e., protection against several pathogens (Bourtzis *et al.*, 2014)). *Wolbachia* were first reported to be found within the reproductive tissues of the *Culex pipiens* mosquito in 1924 by Hertig and Wolbach (Hertig *et al.*, 1924). It is now estimated that *Wolbachia* infect up to 40% of arthropod species (Zug *et al.*, 2012).

Interestingly, the *Wolbachia* genome is not reduced as is seen in other obligatory endosymbionts such as *Buchnera*. The genome sizes range from 1-1.7 Mb. There is a high number of mobile and repetitive elements, with a significant portion encoding ankyrin domains (ANK). Although less common in bacteria, these ANK repeats are thought to mediate host/pathogen protein interactions. *Wolbachia* also have phage particles which actively transcribe and express some elements through a lytic cycle. These phage particles may play a role in introducing and spreading ANK genes, as some ANK genes have been found integrated in prophage segments (Werren *et al.*, 2008).

The *Wolbachia* genome also shows remarkable genetic diversity. There are a large number of genomic differences across even closely related *Wolbachia* strains (Baldo *et al.*, 2010; Siozios *et al.*, 2013). Indeed, it has been suggested that *Wolbachia* is one of the most highly recombining intracellular bacterial genomes known to date (Baldo *et al.*, 2006; Klasson *et al.*, 2009).

Wolbachia comprise a single monophyletic group of related strains which cluster, based upon genetic similarity, into several distinct major clades known as supergroups. The various supergroups tend to be confined to a particular class of hosts. Supergroups C

and D are mostly found in filarial nematodes. Supergroups A, B, E, F, G, and H are found in arthropods, with A and B being the most common. The nematode *Wolbachia* strains have developed a mutualism, where they are obligate reproductive symbionts. This is reflected in the concordance between host and bacterial phylogenies. The arthropod *Wolbachia* strains, however, have no concordance between host and bacterial phylogenies, indicating extensive lateral movement (Werren *et al.*, 2008).

1.2.1 *Wolbachia* utilize host cellular machinery to promote vertical transmission

The primary mode of *Wolbachia* transmission is vertical, from mother to offspring, similar to mitochondrial inheritance. This vertical transmission is achieved through colonization of the germline, and passage to the next generation through the egg cytoplasm. It has been shown in *Drosophila* that *Wolbachia* utilize the host cellular machinery to shuttle within host cells. In the embryo, *Wolbachia* strain *w*Ri (native to *Drosophila simulans* Riverside) was found to colocalize with centrosomal microtubules throughout the cell cycle, and localize near spindle poles and centrosomes during mitosis. Through this localization, *Wolbachia* segregate equally to each spindle pole during mitosis, allowing for equal distribution of *Wolbachia w*Ri throughout the developing embryo (Kose *et al.*, 1995). During early stages of oogenesis in *D. melanogaster* (*D. mel*), *Wolbachia* localize to the anterior of the oocyte, and this localization can be disrupted through manipulations of the host microtubule network (Ferree *et al.*, 2005). In later stages of oogenesis, *Wolbachia* are localized to the posterior of host oocytes, where the germ cells are formed during embryogenesis. This localization requires Kinesin-1, indicating that *Wolbachia* recognition

of host cellular machinery promotes germline-based bacterial transmission (Serbus *et al.*, 2007).

1.2.2 *Wolbachia* induce reproductive manipulations on their host

Wolbachia strains infecting arthropods are well known for the reproductive effects they impart on their hosts, ultimately resulting in their own spread by favoring infected females. The most common of these is cytoplasmic incompatibility, first described by Ghelelovitch and Levin in the 1950s when they discovered a cytoplasmic factor that caused reproductive incompatibility between different strains of *Culex* (terming this cytoplasmic incompatibility, CI). Later, it was found that CI was linked to a Rickettsial agent through antibiotic curing (Werren, 1997). In general, CI is a consequence of *Wolbachia* modification of sperm during spermatogenesis, causing embryonic lethality of uninfected eggs fertilized by sperm from infected males (reviewed by Werren, 1997). Although the precise mechanism is not well understood, the basic premise is that the sperm from infected males is modified (mod^+) and an infected egg with the appropriate rescue factor (resc^+) is required for embryo viability (Tram *et al.*, 2002; Pinto *et al.*, 2013). Extensive analyses of *Wolbachia* population dynamics and localization during spermatogenesis have demonstrated that the density of *Wolbachia* within a whole testis could be the factor determining the modification of the sperm rather than specific infected cysts within a testis (Clark *et al.*, 2003; Riparbelli *et al.*, 2007). Data for *w*Ri show that although there is almost complete CI, there are several uninfected fully elongated spermatid cysts in newly eclosed males. Based on these data, it is believed that CI is a non-cell autonomous effect caused by a diffusible *Wolbachia* factor during spermatogenesis (Riparbelli *et al.*, 2007).

Furthermore, Clark *et al.* (2003) have shown that CI is *Wolbachia* strain specific and when introgressed into a different host genetic background, a CI inducing *Wolbachia* will still induce CI. The host genetic background, however, can still affect the degree of CI through influencing *Wolbachia* levels. CI occurs at variable levels across the *Drosophila* genus (Veneti *et al.*, 2003) and has also been shown to be highly dynamic in other genera such as *Culex* (Duron *et al.*, 2012). *Wolbachia* also induce other reproductive manipulations including the conversion of genetic males into functional females (feminization, mostly in isopods), the exclusive production of female offspring by infected females (parthenogenesis, reported in mites, wasps, and thrips), and the death of male embryos during early embryonic development (male killing, reported in butterflies, beetles, and fruit flies) (Werren *et al.*, 2008).

1.2.3 *Wolbachia* is a novel control agent for infectious disease vectors

Wolbachia have recently emerged as a means for controlling the vectors and causative agents of devastating infectious diseases including malaria, Dengue, Chikungunya, Yellow fever, and West Nile Virus. *Wolbachia* can protect insects from pathogen establishment, as well as limit their ability to transmit diseases. This phenotype was first observed in *Drosophila* naturally infected with *Wolbachia*, where flies were protected against fungal and RNA pathogens (Panteleev *et al.*, 2007; Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Subsequently, it was found that when transferred into a mosquito host, *Wolbachia* reduced the establishment of infection in the mosquito as well as the transmission of several pathogens (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Bian *et al.*,

2010; Walker *et al.*, 2011; Blagrove *et al.*, 2012; Bian *et al.*, 2013). These findings have been expanded to several mosquito host species and numerous pathogens including:

1. *Aedes aegypti* (*Ae. aegypti*) infected with *wMel*, *wMelPop*, and *wAlbB* limits infection of Dengue, Chikungunya, West Nile Virus, and yellow fever viruses (Moreira *et al.*, 2009; Bian *et al.*, 2010; Walker *et al.*, 2011).
2. *Aedes albopictus* (*Ae. albopictus*) infected with *wMel* induces resistance to Dengue and Chikungunya (Blagrove *et al.*, 2012; Blagrove *et al.*, 2013).
3. *Culex quinquefasciatus* naturally infected with *Wolbachia*, shows increased titers of West Nile Virus when cured of *Wolbachia* (Glaser *et al.*, 2010).
4. Transient somatic infection of *Anopheles gambiae* with *wMelPop* or *wAlbB* limits *Plasmodium falciparum* infection (Hughes *et al.*, 2011).
5. *wMelPop* reduces *Plasmodium berghei* titers in *Anopheles gambiae* (Kambris *et al.*, 2010).
6. *wAlbB* reduces oocyst and sporozoite levels in *Anopheles stephensi* (Bian *et al.*, 2013).

Wolbachia do not always confer host resistance to pathogens, however protection is most likely to occur in recently established *Wolbachia* infections. Several reports indicate that the pathogen protection phenotype is dependent upon specific *Wolbachia*-parasite-host combinations, since not all combinations will retard parasite development. Several examples include: *Ae. albopictus* naturally super-infected with *wAlbA* and *wAlbB* exhibiting higher titers and greater transmission of Dengue virus (Blagrove *et al.*, 2012); *wPip* protecting *Culex pipiens* against *Plasmodium relictum* induced mortality, increasing host lifespan (Zelev *et al.*, 2012); *wAlbB* increases titers of *Plasmodium berghei* (Hughes *et*

al., 2012); *Ae. albopictus* infected with *wAlbA* or *wAlbB* does not have reduction in Dengue virus replication (Bian *et al.*, 2010), however *Wolbachia* may limit transmission of Dengue through the salivary glands (Mousson *et al.*, 2012); and *Ae. fluviatilis* naturally infected with *wFlu* has increased titers of *Plasmodium gallinaceum* (Baton *et al.*, 2013). These data indicate the importance of the *Wolbachia*-pathogen-host combination in understanding the relationship between *Wolbachia* and pathogen protection.

The mechanism of pathogen protection is uncertain and the mode of action likely differs between parasites. Several hypotheses exist to explain this phenotype, including immune priming, metabolic competition, *Wolbachia* manipulation of host microRNAs, and *Wolbachia* modulation of host autophagy machinery. There are conflicting reports regarding immune priming. *wAlbB* infection in *Ae. aegypti* induces reactive oxygen species (ROS) activation of the Toll pathway, subsequently leading to reduction of Dengue infection (Pan *et al.*, 2012). However, there is speculation that the immune response is a generalized response due to recent *Wolbachia* introduction into a previously uninfected mosquito (Wong *et al.*, 2011; Rances *et al.*, 2012). There is indirect evidence for metabolic competition where the symbiont and the pathogen utilize the same resources. Immunofluorescence assays of *Wolbachia* infected *Ae. aegypti* show a lack of colocalization between *Wolbachia* and Dengue infected cells (Moreira *et al.*, 2009). Furthermore, it was shown that Chikungunya infection reduces *Wolbachia* titers and microbiota composition in naturally infected *Ae. albopictus* (Tortosa *et al.*, 2008). Additional clues into the mechanisms of pathogen protection include a correlation between

Wolbachia densities and virus interference (Osborne *et al.*, 2009; Frentiu *et al.*, 2010; Lu *et al.*, 2012; Osborne *et al.*, 2012; Chrostek *et al.*, 2013).

1.3 *Wolbachia* tropism

1.3.1 Microbial tissue tropism

During their life, all animals host several microorganisms in their tissues (McFall-Ngai, 2002; Dale *et al.*, 2006; Moran, 2007). The preferential colonization of host tissues by bacteria, referred to as tissue tropism, is a fundamental aspect of these host-microbe interactions. For the host, the tissues being targeted are the key factors determining the consequences of infection, which can range from beneficial to harmful (Hentschel *et al.*, 2000; Klemm *et al.*, 2000; Santos *et al.*, 2004). For the bacteria, being in a specific tissue is also important for evading the immune system, establishing infection, and successfully propagating (Akada *et al.*, 2003; Wilkinson *et al.*, 2003; Santos *et al.*, 2004).

The majority of insects are infected with symbiotic bacteria that are stably maintained across generations and have a profound impact in host biology, including their evolution, physiology, reproduction, immunity and development (McFall-Ngai, 2002; Moran, 2007; Gross *et al.*, 2009; Chaston *et al.*, 2010). Usually these are intracellular bacteria that are maternally transmitted to the next generation.

In the case of obligate mutualistic symbiosis, where both the bacteria and the host require the symbiosis, the bacteria are often found in host structures dedicated mainly to support the bacteria (Braendle *et al.*, 2003; Hosokawa *et al.*, 2010). In such interactions, the bacteria typically produce some factor that the host needs for survival, but cannot produce. *Buchnera*, present in aphids, supply their hosts with amino acids that the aphid

cannot synthesize (Braendle *et al.*, 2003). *Buchnera* reside in specialized cells, known as bacteriocytes, dedicated to the maintenance and transmission of the symbiotic bacteria to the next generation, where they will continue to provide nutrients to the host. These cells offer the remarkable opportunity to understand how the novel cell fates evolve from symbiotic interactions (Braendle *et al.*, 2003; Hosokawa *et al.*, 2010). Throughout most of the host life cycle, the vast majority of the bacteria are present almost exclusively in the bacteriocyte (Baumann *et al.*, 1995; Douglas, 1998; Wilkinson *et al.*, 2003). Similarly, *Wolbachia* is an obligate nutritional mutualist to the bedbug *Cimex lectularius*, where it is found exclusively in the bacteriocyte and provides B vitamins to the host to sustain its growth (Hosokawa *et al.*, 2010).

Tissue tropism tends to be less restricted during facultative endosymbiosis (where the host organism does not depend upon the microbe for survival), as demonstrated by the interaction of the intracellular bacteria *Wolbachia* with several of their insect hosts. Because *Wolbachia* are maternally transmitted, their tropism for the female germline is essential for their vertical propagation to the next generation. However, even in cases where *Wolbachia* is an obligate mutualist, infection can be widespread and somatic tissues are often targeted (Hosokawa *et al.*, 2010; Landmann *et al.*, 2010). The mechanisms involved in *Wolbachia* tropism for different tissues within the host are poorly understood.

1.3.2 *Wolbachia* tropism for stem cell niches

1.3.2.1 *Wolbachia* target the SSCN of *D. melanogaster* during recent and maternal infections

It has been shown that *Wolbachia* accumulate in the SSCN of the *D. mel* ovary, during recent infection as well as during long term maternal infections (Frydman *et al.*, 2006). Upon recent infection through microinjection, *Wolbachia* enter the region of the ovary containing the germarium. This initial invasion of the ovary occurs approximately 14 days post injection, indicating that *Wolbachia* cross tissues very slowly. Within the germarium, the major route for *Wolbachia* to enter the germline in this artificial infection model is through the somatic stem cell niche (SSCN, Figure 1.2, light blue cells). The SSCN is the microenvironment that harbors the somatic stem cell (Figure 1.2, dark blue cells), which in turn generates the somatically derived follicle cells that envelope the germline and secrete the eggshell. This observation in *D. mel* raised the possibility of tropism for stem cell niches as a mechanism to facilitate reaching the germline during horizontal infection.

The same work also showed that *Wolbachia* accumulate at the SSCN in maternally infected flies. These observations and subsequent work in other invertebrates (Hosokawa *et al.*, 2010; Sacchi *et al.*, 2010; Landmann *et al.*, 2012) suggest that stem cell niche tropism plays a widespread role in germline infection during long-term maternal transmission of *Wolbachia*, in addition to the potential role during horizontal transmission.

1.3.2.2 *Wolbachia* target the GSCN in maternally infected *D. mauritiana*

In another fruit fly species, *D. mauritiana* (*D. mau*), *Wolbachia* target the germline stem cell niche (GSCN, Figure 1.2, green cells) in long-term maternally infected flies (Fast *et al.*, 2011). The GSCN is a somatic structure at the anterior tip of the germarium, composed of terminal filament and cap cells (Figure 1.2; TF, light green; CC, dark green) that support the germline stem cells (GSC, Figure 1.2, yellow cells). The GSCs are the source of the germline cells that develop into the eggs.

1.3.3 Mechanisms of cellular invasion, clues from another intracellular bacteria

The mechanisms of *Wolbachia* tissue tropism are unknown. There are numerous developmental, cellular, and molecular hypotheses which could offer an explanation for how *Wolbachia* accumulate in the stem cell niches of *Drosophila*. One potential mechanism could be invasion of stem cell niches by *Wolbachia*. Other bacterial pathogen models may provide useful insight towards understanding the mechanisms of *Wolbachia* invasion.

Listeria monocytogenes is a facultative intracellular bacteria that causes listeriosis. This well-studied pathogen is utilized as a model for host-pathogen interactions, specifically providing insight into how bacteria invade cells, move intracellularly, and disseminate in tissues (Hamon *et al.*, 2006). *Listeria* extracellular membrane proteins, Internalin A and B, bind to receptors on the host cell. Internalin A, specifically, recognizes E-cadherin on the surface of the cell that it will invade inducing cytoskeletal rearrangements via α - and β - catenin culminating in bacterial uptake (Mengaud *et al.*, 1996). E-cadherin is a transmembrane protein required for the correct formation of

adherens junctions between epithelial cells. The intracellular domain of E-cadherin forms a complex with α - and β - catenin, which is required for the cytoskeletal rearrangements needed for bacterial entry (Lecuit *et al.*, 2000).

1.4 *Wolbachia* manipulation of cellular events during oogenesis

Wolbachia is well known for their manipulation of reproductive events in the hosts they infect, ultimately resulting in the spread of more *Wolbachia* infected individuals. In *D. mau*, *Wolbachia* infected flies lay four times more eggs than their uninfected controls (Fast *et al.*, 2011). The increase in egg production is the result of an increase in GSC division, as well as a decrease in programmed cell death of germline cysts in the germarium. Furthermore, the increase in GSC division is correlated with the infection status of the adjacent GSCN. In *D. mau*, only 60% of the GSCNs are highly infected with *Wolbachia*. By comparing division rates of GSCs adjacent to highly and lowly infected niches, Fast *et al.* (2011) showed that the increase in division was significantly higher when GSCs are adjacent to highly infected niches. Because the highly and lowly infected niches occur within the same fly and ovary, the host factors and internal host environment were a constant, allowing for the direct comparison of niche tropism with GSC division.

1.5 Dissertation rationale and summary

Symbiotic bacteria are emerging as a promising tool for the control of vector-transmitted infectious diseases. The endosymbiont *Wolbachia* offers novel means for the control of several vector-transmitted pathogens, including Dengue, West Nile Virus, Chikungunya, and *Plasmodium*. Recent work, however, shows that *Wolbachia* mediated protection against pathogens is not a universal phenotype (Hughes *et al.*, 2014) and it is

essential to understand the molecular mechanisms involved. Although arthropod vectors form associations with microbial symbionts in virtually every environment, our knowledge of the mechanisms of these host-symbiont-pathogen interactions are limited. Understanding the cellular and molecular bases of tissue tropism is a fundamental aspect of *Wolbachia*-host interactions.

In Chapter 3, we investigated the evolutionary mechanisms of stem cell niche tropism in the *Drosophila* ovary. A survey of the *Drosophila* genus revealed that stem cell niche tropism is an evolutionarily conserved phenotype. Furthermore, we found that the mechanisms involved in niche tropism in the ovary were *Wolbachia* derived, and that stem cell niche tropism facilitates vertical transmission. Chapter 4 follows up experiments performed in the ovary, investigating the conservation of stem cell niche tropism in *Drosophila* males. We show great disparity in the evolutionary conservation and underlying mechanisms in stem cell niche tropism between male and female gonads. In contrast to females, niche tropism in the male testis is not pervasive. Furthermore, both *Wolbachia* and host derived factors play roles in the targeting of the stem cell niche in the testis, the hub. Chapter 5 focuses on the cellular and molecular mechanisms of stem cell niche tropism in the *D. mel* testes. Identified in Chapter 4, several strains of *Wolbachia* present in *D. mel* target the hub at varying frequencies and densities. The hub is a well characterized structure developmentally, cellularly, and molecularly, allowing for the careful multi-level analysis of mechanisms involved in stem cell niche tropism. We have determined that *Wolbachia* target the hub early in development, and accumulate throughout host development via preferential replication inside the hub cells. Furthermore, we have

found that the host protein Armadillo (β -catenin) facilitates this accumulation, potentially through the Wnt signaling pathway.

Figure 1.1 The *Drosophila* female anatomy

A. Female fruit fly with approximate location of the ovary. B. Diagram of the ovaries. Each female fly has two ovaries comprised of several strings of developing egg chambers called ovarioles. The mature eggs are deposited through the oviduct, where they become fertilized by sperm stored in the spermatheca. C. Diagram of the ovariole. At the anterior tip of the ovariole is the germarium, housing the stem cells (See Figure 1.2). Development proceeds from left to right of the diagram, from anterior to posterior. Egg chambers, comprised of 15 interconnected nurse cells and an oocyte surrounded by a follicular epithelium exit the germarium and proceed through 14 stages of oogenesis until the mature egg is fully developed. Adapted from (Mahowald *et al.*, 1980),(Frydman *et al.*, 2001), and (Hartenstein, 1993).

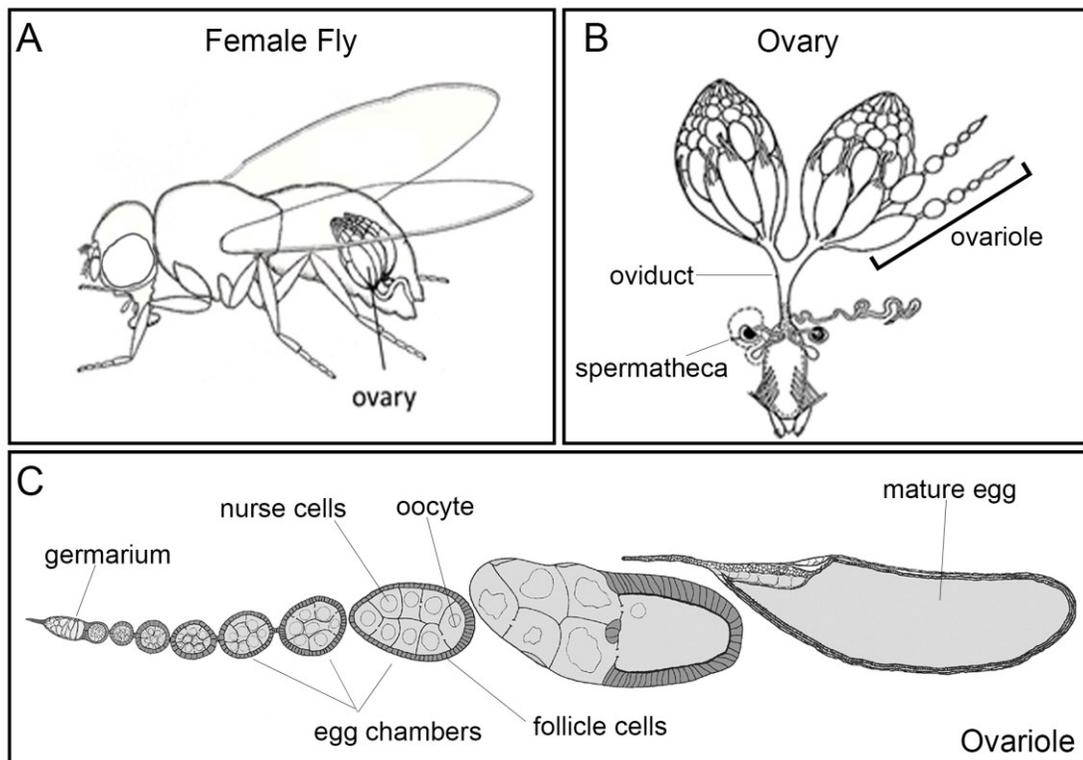


Figure 1.2 Diagram of the germarium

The germarium is located at the anterior tip of the ovariole and houses the cells necessary to form an egg. The germline stem cells (GSC, yellow) are anchored to the GSC niche (GSCN). The GSCN is comprised of the cap cells (dark green) and terminal filament (light green). The GSCs divide asymmetrically, with one cell remaining at the niche as the GSC and the other cell exiting the niche as a germline daughter cell. The daughter cell then undergoes four rounds of division with incomplete cytokinesis to produce a germline cyst (red). The escort cells (gray) guide the germline cysts through the germarium until they are enveloped by the follicle cells. The somatic stem cell (SSC, dark blue) divides asymmetrically with one cell remaining anchored to the SSC niche (SSCN, light blue) and the other exiting the niche. The somatic daughter cell undergoes transient amplification and migrates to surround the germline cysts. Adapted from (Frydman *et al.*, 2006).

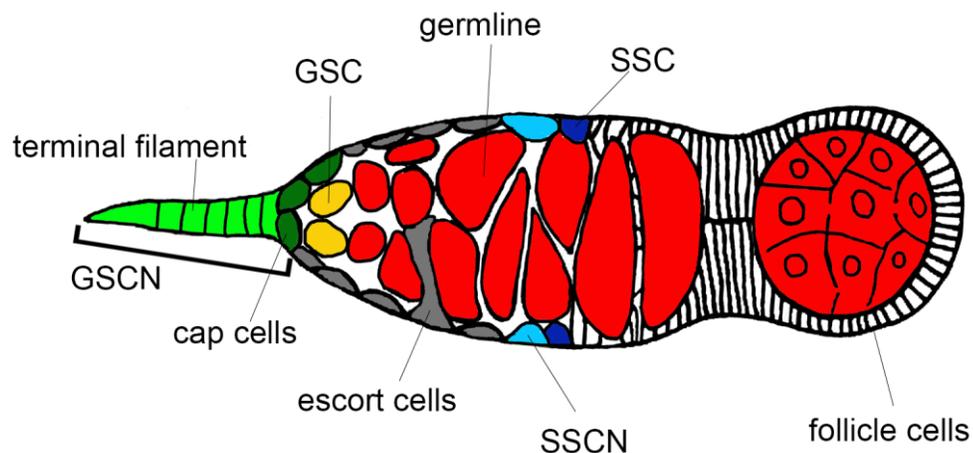


Figure 1.3 The *Drosophila* male anatomy

A. The male fruit fly with approximate location of the testes. B. Diagram of the testes. The stem cells and niche are located at the apical tip of the testis lobe. Spermatogenesis progresses through the testis lobe. As the sperm individualize and become motile, they cross through the seminal vesicle and are stored in the accessory glands. C. Diagram of the apical tip of the testis. The germline stem cells (GSCs) are each surrounded by a pair of cyst stem cells (CySCs) and are arranged radially around the hub (green, stem cell niche). The GSCs and CySCs divide asymmetrically with one set of cells remaining as the stem cells and the others forming a cystoblast. Adapted from (Patterson, 1943) and (de Cuevas *et al.*, 2011)

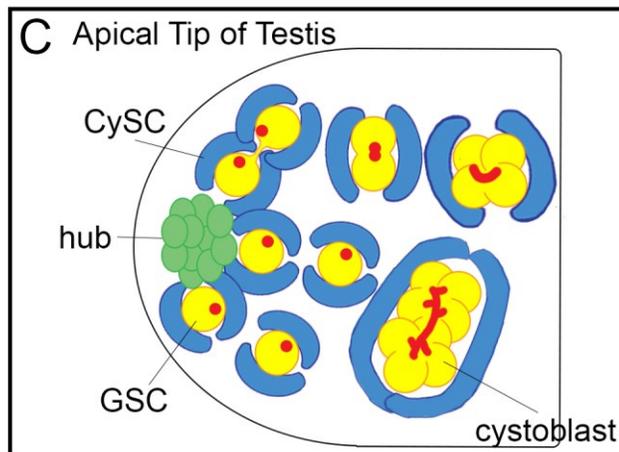
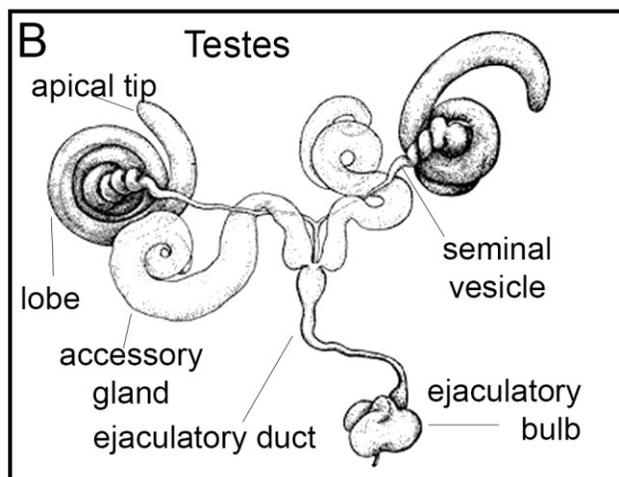
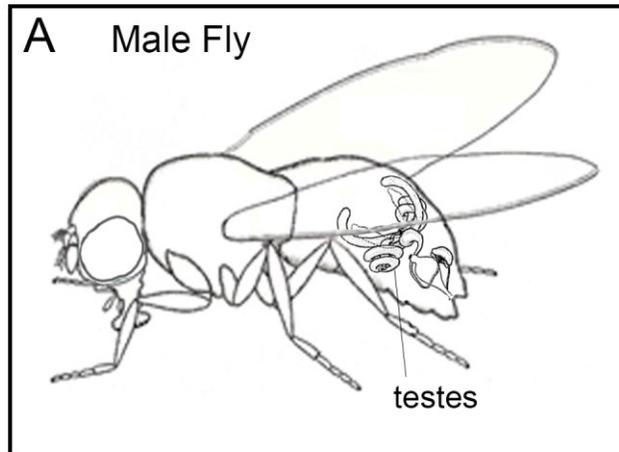
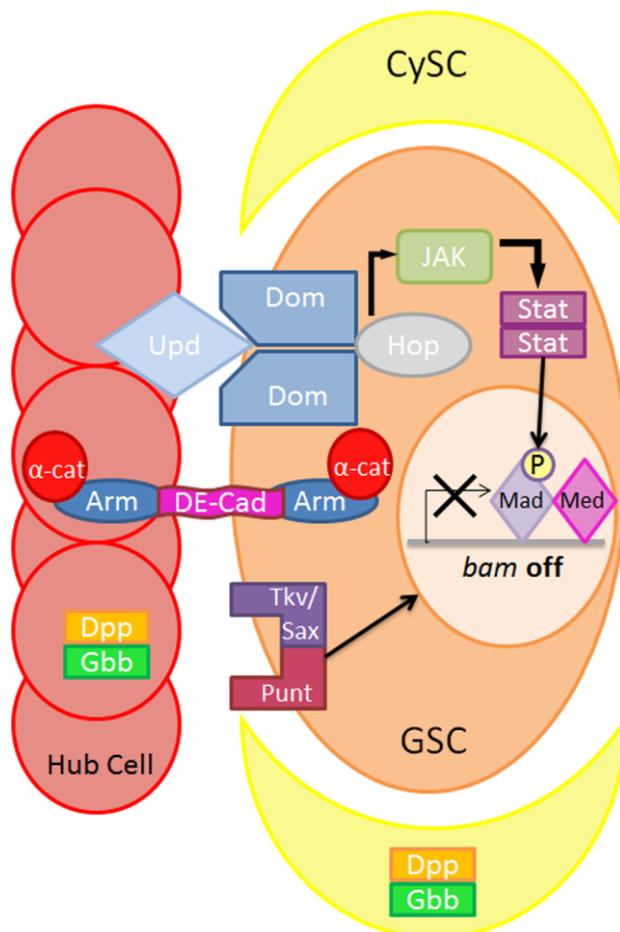


Figure 1.4 Stem cell signaling in the testis

The hub secretes the JAK-STAT ligand Unpaired, which binds to the receptor, Domeless, on the germline stem cells. The signaling cascade propagates through Hopscotch, Jak, and STAT, resulting in the repression of the differentiation gene *bam*. BMP signaling molecules Dpp and Gbb also contribute to the suppression of *bam*. Stem cells are anchored to the hub via Adherens junctions, formed by DE-Cadherin, Armadillo, and α -catenin. Adapted from (Wong *et al.*, 2005).



CHAPTER 2 Materials and Methods

2.1 Fly husbandry and stocks used for analysis

Flies were raised at room temperature and fed a typical molasses, yeast, cornmeal, agar food, with the exception of the following: *D. sechellia* flies were supplemented with reconstituted Noni Fruit (Hawaiian Health Ohana, LLC)(Amlou *et al.*, 1998); *D. innubila* flies were raised on Instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC) supplemented with a mushroom (Dyer *et al.*, 2004). All fly stocks with their infection statuses and sources are shown in Table 2.1.

2.2 Raising of adult cultures

2.2.1 *Wolbachia* stem cell niche tropism

Newly eclosed flies were collected and raised at room temperature, fluctuating around 22°C with no humidity control, for one week. Flies were dissected on day seven.

2.2.2 GAL4-UAS genetic crosses

Unless otherwise noted, virgin females were collected from room temperature stocks and crosses were kept in a 25°C incubator with 60% humidity. Flies of the proper genotype were collected and aged to the appropriate age at 25°C with 60% humidity.

2.2.3 Stem cell division

Crosses with forty females and forty males were set up in bottles from young flies kept at room temperature and placed at the appropriate temperature (25°C or 29°C) to lay eggs. Progeny were allowed to develop at the experimental temperature and newly eclosed flies were collected for either dissection or aging for 7 days, at the experimental temperature.

2.2.3.1 CO₂ effects on stem cell division

To assess the effect of CO₂ on stem cell division, flies were either subjected to rapid or extended CO₂ exposure. For the rapid exposure, adult flies were counted and separated into vials containing no more than 5 males and 5 females. Upon dissecting, flies were left on the CO₂ fly pad for no more than 2 min. Conversely, for the extended CO₂ exposure, adult flies were left on the CO₂ fly pad for approximately 1 h and then dissected.

2.3 Microdissection of tissue from *Drosophila* adults

Adult ovaries and testes were dissected in either plastic or glass dissection wells in Grace's media. Unless otherwise noted, within 20 min of dissection, tissue was fixed for 20 min in 4% paraformaldehyde (PFA, EM grade), 0.2% Triton X-100 and Graces. Fix was removed with three washes in PBS containing 0.2% Triton X-100 (PBT). Tissue was stored in PBT containing 0.2% bovine serum albumin (BSA), 5% normal goat serum (NGS) and 0.005% sodium azide (PBANG) at 4°C.

2.4 Immunofluorescence microscopy

2.4.1 General immunostaining

Tissue was blocked for at least 1 h with PBANG before incubation with primary antibodies (for dilutions, see Table 2.3). Incubation with primary antibody (diluted in PBANG) was conducted for 2-4 h at room temperature, or overnight at 4°C, nutating. Following incubation, the primary antibody was removed and saved for re-use (up to three times, depending on the antibody). The tissue was quickly washed three times with PBT, followed by three 40 minute washes with PBT containing 0.2% (w/v) BSA (PBT/BSA), nutating. The tissue was further blocked with PBANG for 30 min. The tissue was then

incubated with secondary antibody (diluted in PBANG, for dilutions, see Table 2.3) for 2 h at room temperature, nutating, in the dark. Following incubation, the secondary antibody was removed and saved for re-use (up to 3 times) and the tissue was quickly washed three times with PBT. The tissue was then washed for 2 additional hours in PBT/BSA, nutating, in the dark. To label nuclei, tissue was incubated for 30 min in 10 $\mu\text{g}/\text{mL}$ Hoechst (Life Technologies) at room temperature, nutating, in the dark. After removal of the Hoechst, the tissue was quickly washed two times with PBT/BSA and then two times with PBS. Tissue was then mounted in Prolong Gold (Life Technologies). After mounting media had sufficient time to polymerize (usually overnight), the coverslips were sealed with nail polish.

2.4.2 *In situ* hybridization (ISH)

2.4.2.1 ISH on dissected tissues

Protocol was adapted from (Heddi *et al.*, 1999; Moreira *et al.*, 2009).

Tissue was dissected in Graces and fixed in 4% PFA solution with 0.2% Triton X-100 and Graces. Specific oligonucleotide probes labeled with Cy3 at the 5' end were designed against the 16SrRNA of *Wolbachia* (Integrated DNA Technologies, Table 2.4).

Hybridization was performed at 37°C in 50% Formamide (v/v), 5x SSC, 250 mg/l Salmon sperm DNA, 0.5x Denhardt's solution, 20mM Tris-HCl, and 0.1% SDS (w/v). After a 30 min pre-incubation period, tissue was incubated in 100ng of each for 3 h. Tissue was then washed twice for 15 min at 55°C in a 1x SSC wash with 0.1% SDS and 20 mM Tris-HCl and then twice for 15 min in a 0.5x SSC wash with 0.1% SDS and 20 mM Tris-HCl. Hoechst was added to the second 0.5x SSC wash at a concentration of 10 $\mu\text{g}/\text{mL}$.

2.4.2.2 *in situ* hybridization on whole mosquitoes

Protocol was adapted from (Koga *et al.*, 2009).

Whole mosquitoes with wings and legs removed (for penetrance and convenience), were placed in Carnoy's solution [6 Ethanol (EtOH): 3 Chloroform: 0.5 Acetic Acid] for 24 h, nutating at room temperature. Mosquitoes were washed three times quickly with EtOH and abdomens were cut off or poked if desired. Mosquitoes were then placed in a 6% alcoholic H₂O₂ solution for 3 to 10 days to reduce autofluorescence. After quenching, mosquitoes were then washed three times quickly in 100% EtOH and then incubated for 30 min in 100% EtOH, nutating. Mosquitoes were then slowly rehydrated with PBT and subjected to the ISH protocol.

2.4.3 Dual *in situ* hybridization and immunostaining of tissue

Tissue was dissected immediately before immunostaining was performed to reduce the chances of mRNA degradation. All steps of immunostaining were conducted without serum, as the RNases and DNases could degrade the mRNAs. Also, all *in-situ* reagents and buffers were diethylpyrocarbonate (DEPC) treated to eliminate RNases which would otherwise degrade the target RNAs. Tissue was fixed for 20 min in 4% PFA, 0.2% Triton X-100 and Graces. Fix was removed and tissue was washed three times with PBT. Tissue was incubated in primary antibody (diluted in PBT) for 2 h at room temperature, nutating. Primary antibodies were used in concentrated form that could be diluted at least 1:1000 to reduce the chances of signal degradation due to RNases in the antibody solution. Primary antibody was removed and the tissue was quickly washed three times with PBT and then for 1.5 h with PBT, nutating. Tissue was then incubated for 1.5-2 h in secondary antibody

(diluted in PBT), nutating, in the dark. Secondary antibody was removed and the tissue was quickly washed three times with PBT then for an h with PBT, nutating. The tissue was then fixed again for 30 min with 4% PFA, 0.2% Triton X-100, and Graces and washed three times with PBT.

Tissue was then pre-hybridized for 30 minutes at 37°C and hybridized overnight with 100 ng of each probe at 37°C. The remainder of the *in situ* protocol was performed as written in Section 2.4.2.1, the following day.

2.4.4 *Wolbachia* antibody staining controls

Most of the *Wolbachia* antibody staining is utilizing an antibody against Hsp60. To test the specificity of this antibody, it was used on W- ovaries (Figure 2.1A) and testes (Figure 2.1C). Antibody staining was also compared to *in situ* hybridization with *Wolbachia* specific probes (See Table 2.1) of ovaries (Figure 2.1B) and testes (Figure 2.1D).

2.5 Identification of stem cell niches for tropism analysis

The SSCN and associated somatic stem cells (SSCs) reside at the boundary between regions 2a and 2b of the germarium (Figure 1.2). For the purpose of this analysis, this boundary was defined as the border region (BR), encompassing the SSCN and SSC, as previously done (Frydman *et al.*, 2006). Association with the adjacent somatic stem cell identified by lineage labeling is the most reliable method to identify the stem cell niche (Fox *et al.*, 2009). Due to the general lack of genetic and cytological SSC and SSCN markers across the *Drosophila* genus, somatic stem cell niche tropism was considered as a more general tropism for the somatic tissue at the border region.

Germline stem cell niche tropism consists of tropism to two main cell types comprising the GSCN: the cap cells (CC) and the terminal filament (TF) cells (Figure 1.2).

2.6 Image analysis of *Wolbachia* niche tropism

2.6.1 Visual identification of niche tropism

Presence of fluorescent labeling for *Wolbachia* in the GSCN, SSCN, or hub was visually identified and counted using epifluorescence at 600x magnification using Olympus Fluoview 1000 Confocal microscope. Representative images of niche tropism for each species were acquired and visual identification of niche tropism was confirmed in a subset of representative confocal images using MatLab software for image processing.

2.6.2 *Wolbachia* density analysis in the ovary

Z stacks of representative images were analyzed for *Wolbachia* density in the soma and germline in several regions of the germarium using MatLab software, as defined by Frydman, *et al.* 2006. *Wolbachia* in the soma and germline were distinguished via overlap with Vasa marking the germline. Manual masks were drawn to separate the following regions of the germarium: GSCN, 1, 2a, border region, 2b, and 3. The GSCN was considered separately from region 1. Manual corrections were applied for unclear or ambiguous Vasa staining.

2.6.3 Quantification of *Wolbachia* tropism in the ovary

GSCN and SSCN tropism was assessed relative to *Wolbachia* density in the somatic cells of region 2b as a base level of *Wolbachia* in the soma. Region 2b was chosen based on overall consistent levels of *Wolbachia* across species and because differentiating between germline and soma based on Vasa staining is the most consistent in this region.

Infection of the stem cell niche was considered tropism if the relative levels were increased by at least 1.5 fold.

2.6.4 *Wolbachia* tropism analysis in the testis

Z stacks of representative images were analyzed for *Wolbachia* density in the hub and surrounding tissue using immuno-markers to label the hub and MatLab software. Manual masks were drawn around the hub and surrounding soma and germline to obtain measurements of the relative *Wolbachia* levels. Infection of the hub was considered tropism if the relative levels in the hub were increased by at least 1.5 fold as compared to the surrounding tissue.

2.7 Introgression crosses

Introgression crosses were performed according to Figure 2.2. Female flies with the *Wolbachia* strain of interest were backcrossed for five generations to males with the genetic background of interest. To confirm the introgression, the morphology of the male genital arch was observed, which is genetically controlled by approximately 40 loci scattered throughout the genome (Macdonald *et al.*, 1999).

2.8 Phylogenetic Analyses

To quantify the correlation of niche tropism pattern in either the ovary or testis to the *Wolbachia* or *Drosophila* phylogenies, we utilized a computer simulation model of randomized character distributions to compare with the distribution of niche tropism pattern on each of the phylogenies (Maddison *et al.*, 2005). We used tree length as a measurement for goodness of fit for the distribution of a character, such as the tropism pattern, as aligned with the phylogeny. Tree length is defined as the total number of steps

required to map a data set onto a phylogenetic tree. By comparing the tree lengths between tropism pattern aligned to both phylogenies, we could determine which phylogeny was a better fit for the data, allowing an inference into which organism the trait evolved in.

2.9 FtsZ analysis of *Wolbachia* division

In dividing bacteria, FtsZ creates a ring structure during septation and is required through the final step of division. In non-dividing bacteria, FtsZ is not localized and is distributed throughout the bacterial cell (Weart *et al.*, 2003; Landmann *et al.*, 2010). Thus, by quantifying the localization of FtsZ in each *Wolbachia* cell, we can assess the number of dividing *Wolbachia*. For a precise measurement it is important to determine the distribution of FtsZ within each individual *Wolbachia*. It would be difficult to assess distribution of FtsZ in situations where *Wolbachia* densities are high. Therefore, experiments were conducted at developmental time points and in species with relatively low *Wolbachia* densities, where single *Wolbachia* are more prevalent.

2.10 Tetracycline treatment to assess *Wolbachia* tropism mechanism

Crosses with fifty female and fifty male flies were set up in triplicate in bottles containing normal fly food and placed at 25°C, with 60% humidity. After 3 days of egg laying, adults were transferred into bottles containing food with 0.05 mg/mL tetracycline, prepped the previous day. Adults were allowed to lay eggs for 3 days before being removed. Larvae developed in the presence or absence of tetracycline until eclosion. For the non-tetracycline treated larvae, newly eclosed adults were collected and split in half. Half were dissected immediately (newly eclosed, NE-No Tet) and half were aged for 7 days in vials containing normal fly food (7d-No Tet/No Tet). For the tetracycline treated

larvae, the newly eclosed adults were split into three samples. One sample was dissected immediately (NE-Tet), one sample was aged for 7 days in vials containing normal food (7d Tet/No Tet), and the third sample was aged for 7 days in vials containing food with 0.05 mg/mL tetracycline (7d Tet/Tet). A schematic of this experiment is shown in Figure 2.3.

2.11 Identification of *Wolbachia melanogaster* subtypes

It was previously thought that there was only a single *Wolbachia* infection of *Drosophila melanogaster*. Since the *wMel* genome sequence was completed, several polymorphic markers have been identified within the *wMel* genome that can be used to discriminate among five different *Wolbachia* variants. These markers include a chromosomal inversion, IS5 transposon insertions sites and variable number tandem repeats (VNTRs). *wMelPop* and *wMelCS* can be distinguished based upon the number amplifications of the ‘octomom’ region, where *wMelPop* has 8-10 copies of this region and *wMelCS* has only 1 (Chrostek *et al.*, 2013). Table 2.5 summarizes the variable markers utilized to distinguish the three strains of *Wolbachia* present in our lab. Table 2.6 summarizes the diagnostic flanking PCR primers used. Figure 2.4 displays the typing results for our *wMel* strains.

2.12 GAL-UAS System

The GAL4-UAS system can be used in *Drosophila* for targeted gene expression in a tissue specific manner (Brand *et al.*, 1993). Identified in yeast, GAL4 encodes a protein of 881 amino acids which binds to a 17 base pair site, defined as an upstream activating sequence (UAS), analogous to enhancers in multicellular eukaryotic organisms. The UAS sequence is essential for the transcription of GAL4-regulated genes in yeast. This system

is versatile, allowing for expression of particular genes in specific tissues using a variety of tissue specific GAL4 promoters (Figure 2.5). Additionally, this system can be used to drive transcription of mutant transcripts and RNAi hairpins, resulting in a knockdown of endogenous gene expression. In *Drosophila*, the GAL4 drivers and the UAS element are maintained in separate fly lines and the UAS element becomes activated in the progeny when the two lines are mated together (Duffy, 2002). The generic fly crosses utilized in the majority of the GAL4-UAS studies are demonstrated in Figure 2.6.

2.13 Statistical Analysis

For all P-values listed, the statistical test used is indicated. Statistical tests were performed using Microsoft Excel or R.

Figure 2.1 *Wolbachia* antibody staining controls

A. Antibody staining of a *Wolbachia* uninfected (W-) control. Hub marker in red, DNA in blue, Hsp60 staining of *Wolbachia* in green. Little background staining occurs in a W-control. **B.** In situ hybridization for *Wolbachia*. DNA in blue, a DNA probe against the *Wolbachia* 16S-rRNA is in green. **B'**. Gray scale inset of *Wolbachia* channel in the hub. **B''**. Gray scale inset of DNA in the hub. **C.** Hsp60 antibody staining of *Wolbachia* infected testis. **C'**. Gray scale inset of the *Wolbachia* channel only. **C''**. Gray scale inset of DNA channel only. The inset shows haze of DNA stain for *Wolbachia* in the hub, along with brighter spots of A/T rich regions of host nuclear DNA (usually heterochromatic regions).

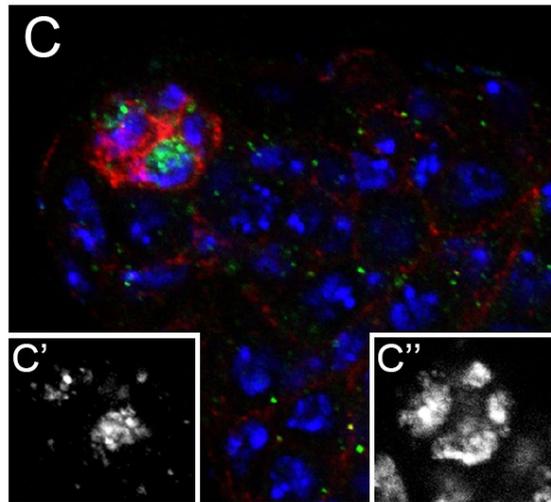
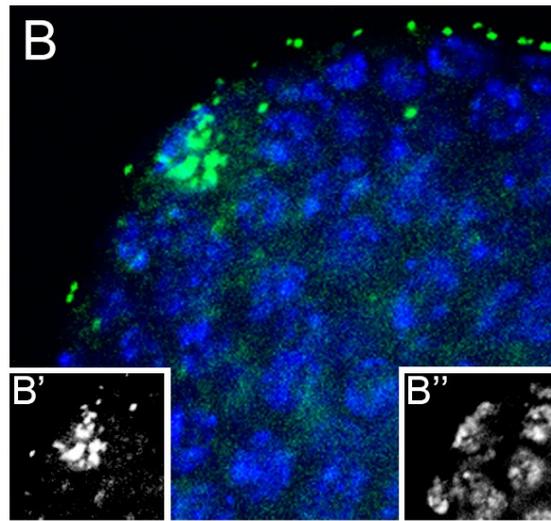
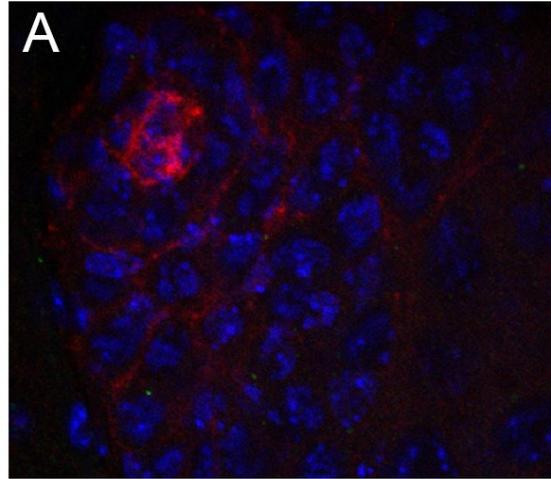


Figure 2.2: Diagrams of experimental design and genetic introgression

A. Schematic of genetic introgression. Female flies of species A carrying *Wolbachia* A are backcrossed to males of species B for 5 generations to introgress the species B genetic background into a fly carrying *Wolbachia* A. **B.** Diagram illustrating experimental design of the hybrid cross to introgress *Wolbachia* A into species B genetic background to determine whether *Wolbachia* factors or *Drosophila* factors have a greater influence on niche tropism pattern.

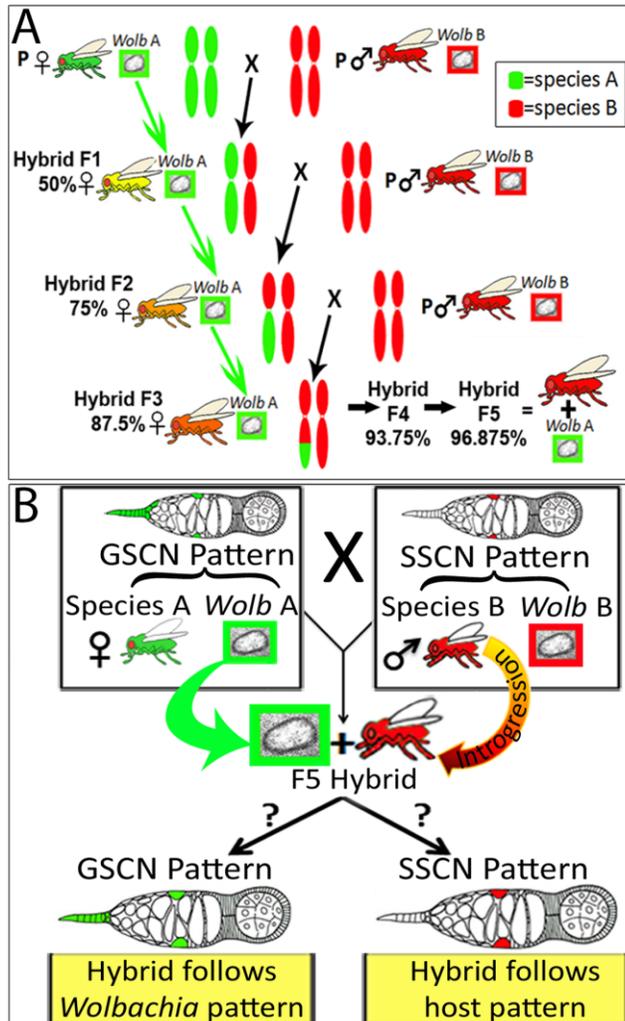


Figure 2.3: Tetracycline experimental setup

A. Schematic of experimental set up. 50 male and 50 female flies were set up in a bottle of fly food with no tetracycline. After four days, adult flies were flipped in bottles with 0.05mg/mL Tet in the food. Eggs/larvae were allowed to develop on the food with and without tetracycline. Upon eclosion, flies were collected. For non-tetracycline treated flies, half of the progeny were dissected at newly eclosed (NE-No Tet) and half were aged to seven days on food without tetracycline (7d- No Tet/No Tet). Flies that were raised on tetracycline were split into three groups: The first was dissected immediately (NE- Tet); The second group was aged to 7 days without tetracycline (7d- Tet/No Tet); The third group was aged to 7 days on 0.05mg/mL tetracycline (7d- Tet/Tet). **B.** Timeline of treatment for each experimental group. Blue bars indicate tetracycline in food. White bars indicate no tetracycline in food.

Figure 2.4: Identification of *w*Mel strains

A. Different *w*Mel strains can be distinguished by the number of variable number tandem repeats (VNTRs) and by the location of an IS5 insertion. Strains are identified according to criteria from (Riegler *et al.*, 2005) and (Ilinsky, 2013): 1. *w*Mel2 (original infection; 5 repeats for VNTR-105, 6 repeats for VNTR-141, IS5 at WD016/7) 2. *w*MelCS (original infection; 4 repeats for VNTR-105, 6 repeats for VNTR-141, IS5 at WD1310) 3. *w*Mel (original infection; backcrossed into W- background; 5 repeats for VNTR-105, 7 repeats for VNTR-141, IS5 at WD016/7) 4. *w*Mel (backcrossed into W- background; 5 repeats for VNTR-105, 7 repeats for VNTR-141, IS5 at WD016/7) 5. *w*MelCS (backcrossed into W- background; 4 repeats for VNTR-105, 6 repeats for VNTR-141, IS5 at WD1310). **B.** Octomom amplification diagnostic. WD0505 and WD0519 are outside of the octomom region and should have no repeats in any sample. WD0509 and WD0512 should be amplified in *w*MelPop only. Control flies: *w*Mel (light blue) and *w*MelCS (gray) have no amplification at this region and *w*MelPop (orange) has 8-10 repeats in this region. Frydman lab *w*Mel (dark blue) has no repeats as expected. Frydman lab strain #7 is *w*MelCS as indicated by lack of repeats in this region.

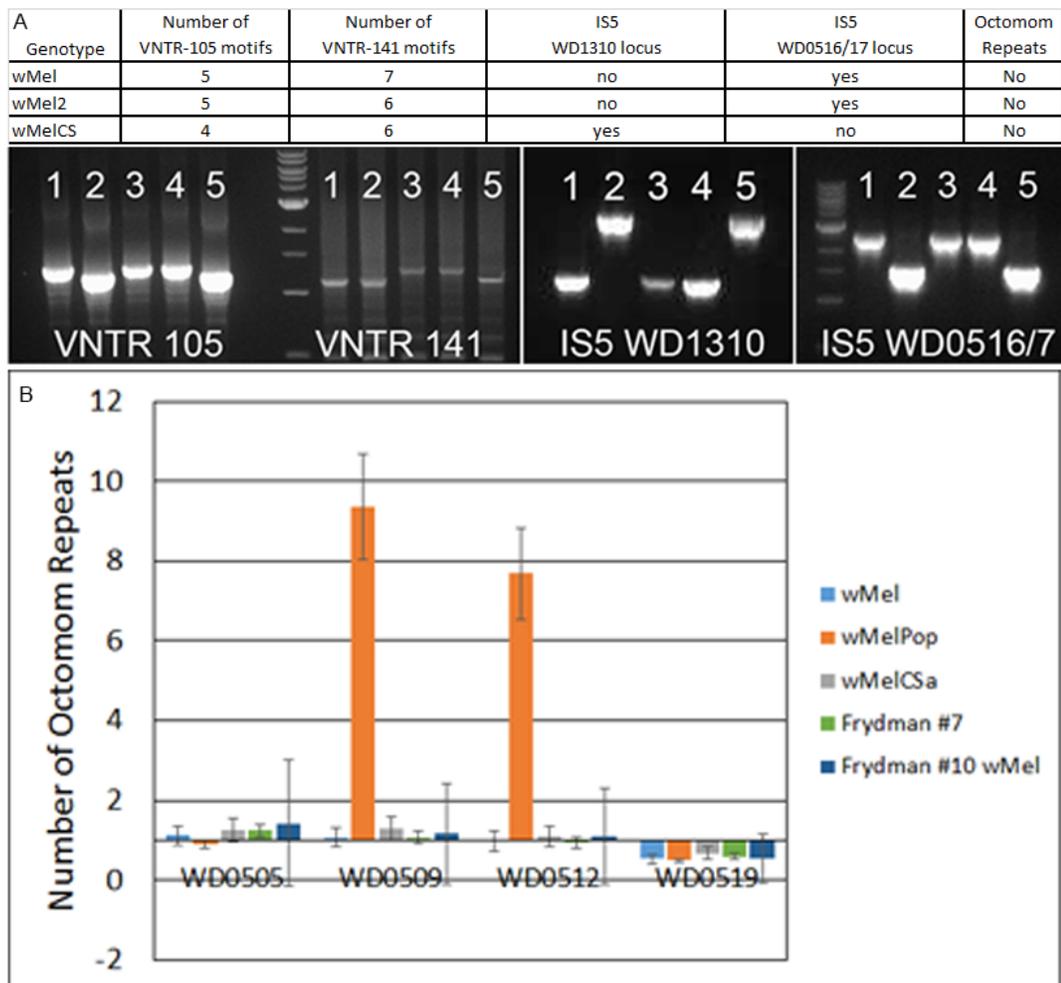


Figure 2.5 The GAL4-UAS system

A. General scheme for GAL4-UAS system. A tissue specific promoter drives expression of GAL4. The GAL4 protein binds to the upstream activating sequence (UAS) to drive expression of a gene of interest (G.O.I.). **B.** To drive RNAi with the GAL4-UAS system, the G.O.I. is an inverted repeat complementary to the mRNA being targeted for knockdown. The inverted repeat forms a hairpin double-stranded RNA which enters the endogenous RNAi pathway.

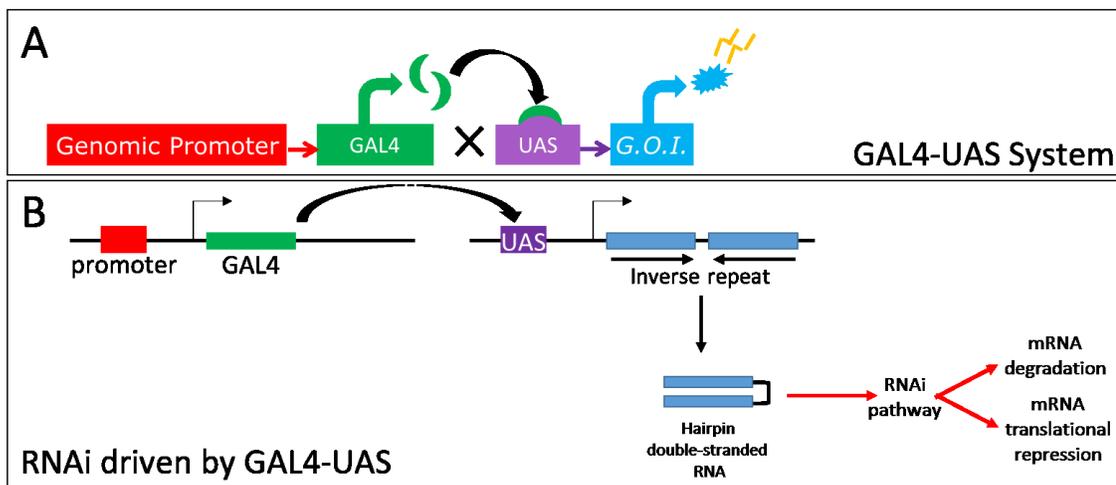


Figure 2.6 Generic GAL4-UAS cross scheme

Cross scheme for RNAi construct on the 3rd chromosome. A similar scheme was used if the RNAi construct was on the 2nd chromosome. If the construct was already balanced, then only the second cross was needed to obtain the flies used for analysis.

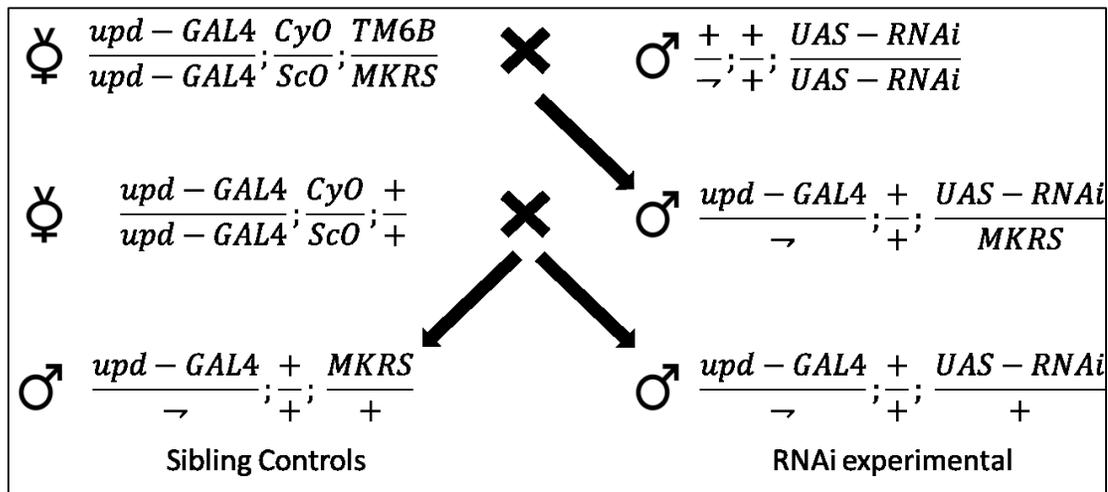


Table 2.1 Sources for *Drosophila* species used for analysis

<i>Drosophila</i> Species	<i>Wolbachia</i> Strain	Source	Frydman Stock #	Stock Center #/ Reference
<i>D. melanogaster</i>	wMel	Frydman Lab	10, 200	–
<i>D. melanogaster</i>	wMelCS	Sullivan Lab	7, 201	–
<i>D. simulans</i>	wNo	San Diego Stock Center	33	14021-0251.198
<i>D. simulans</i>	wRi	San Diego Stock Center	42	14021-0251.169
<i>D. sechellia</i>	wSh	San Diego Stock Center	41	14021-0248.08
<i>D. mauritiana</i>	wMau	San Diego Stock Center	24	14021-0241.01
<i>D. teissieri</i>	wTei	San Diego Stock Center	37	14021-0257.00
<i>D. yakuba</i>	wYak	Virginie June2008	39	–
<i>D. tropicalis</i>	wWil	San Diego Stock Center	45	14030-0801.01
<i>D. innubila</i>	wDin	John Jaenike	168 (lost)	–
<i>D. ananassae</i>	wAna	Jack Werren/Michael Clark	171	–
<i>D. melanogaster</i>	wMel	Luis Teixeira	441	Chrostek <i>et al.</i> , 2013
<i>D. melanogaster</i>	wMel2	Luis Teixeira	443	Chrostek <i>et al.</i> , 2013
<i>D. melanogaster</i>	wMel3	Luis Teixeira	449	Chrostek <i>et al.</i> , 2013
<i>D. melanogaster</i>	wMelCS	Luis Teixeira	445	Chrostek <i>et al.</i> , 2013
<i>D. melanogaster</i>	wMelCS2	Luis Teixeira	447	Chrostek <i>et al.</i> , 2013
<i>D. melanogaster</i>	wMelPop	Luis Teixeira	442	Chrostek <i>et al.</i> , 2013
<i>D. mauritiana</i>	wSh	Frydman Lab	113	–
<i>D. sechellia</i>	wMau	Frydman Lab	114 (lost)	–
<i>D. simulans</i>	wMel	Kostas Bourtzis via the Sullivan Lab	166	–
<i>D. simulans</i>	wRi	Frydman Lab	143	–
<i>D. simulans</i>	wNo	Frydman Lab	142	–

Wolbachia strains were classified based on previous work (Paraskevopoulos *et al.*, 2006; Chrostek *et al.*, 2013) and multi locus sequence type (MLST) in our lab. Strains in bold are infected with non-native *Wolbachia* strains, introduced by genetic introgressions or embryonic microinjection (Toomey *et al.*, 2013).

Table 2.2: Transgenic fly stocks used for analysis

Shorthand Name	Genotype	Frydman Stock #	Source
Upd-Gal4 Driver	$\frac{upd - gal4}{upd - gal4}; \frac{+}{+}; \frac{+}{+}$	390 W- 391 wMel 392 wMelCS	Frydman Lab
Upd-Gal4 Driver bal on II	$\frac{upd - gal4}{upd - gal4}; \frac{CyO}{ScO}; \frac{+}{+}$	224 W- 225 wMel 226 wMelCS	Frydman Lab
Upd-Gal4 Driver double bal	$\frac{upd - gal4}{upd - gal4}; \frac{CyO}{ScO}; \frac{MKRS}{TM6B}$	368 W- 369 wMel 370 wMelCS	Frydman Lab
Double Balancer	$\frac{+}{+}; \frac{CyO}{ScO}; \frac{MKRS}{TM6B}$	289 W-	McCall Lab
UAS-Armadillo-RNAi	pTRiP[JFO1251]attP2/TM3	363	McCall Lab; BL#31304
UAS-Armadillo-RNAi	pTRiP[JFO1252]attP2	364	McCall Lab; BL#31305
UAS-DEC-RNAi	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL00646}attP40		BL#38207
UAS-DEC-RNAi	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02769}attP2/TM3, Sb[1]		BL#27689
UAS-Upd-RNAi	On III	343	Matunis Lab, VDRC 3282
UAS-Upd OE	w, P(w+, UAS.upd)26.2/CyO	341	Matunis Lab
UAS-Arm ^{s10}	On II	382	Perrimon Lab

Wolbachia strains originated from Frydman stocks #200 (W-), #201 (wMel), and #202 (wMelCS).

Table 2.3 Antibodies used for immunohistochemistry

Name	dilution	host	Source
<i>Primary antibodies</i>			
hsp60	1:100	Mouse	Lk2, Sigma
Vasa	1:5	Rat	DSHB (for use in <i>D. mel</i> only)
Vasa	1:500	Rat	Paul Lasko
Vasa	1:1000	Rabbit	Ruth Lehmann
phosphorylated histone 3	1:200	Rabbit	Upstate Biotech
Lamin C28	1:20	Mouse	DSHB (LC28.26)
Armadillo	1:100	Rabbit	Santa Cruz
Alpha-catenin	1:40	Rat	DSHB (DCAT1)
DE-Cadherin	1:50	Rat	DSHB (DCAD2)
DE-Cadherin	1:100	Rabbit	Santa Cruz
DN-Cadherin	1:20	Rat	DSHB (DN Ex #8)
<i>Wolbachia</i> FtsZ	1:1000	Rabbit	Bill Sullivan
<i>Secondary antibodies</i>			
anti-mouse, alexa 488	1:500	goat	Life Technologies
anti-mouse, alexa 546	1:500	goat	Life Technologies
anti-mouse, alexa 647	1:500	goat	Life Technologies
anti-rabbit, alexa 546	1:500	goat	Life Technologies
anti-rabbit alexa 633	1:500	goat	Life Technologies
anti-rat, alexa 568	1:500	goat	Life Technologies
anti-rat, alexa 647	1:500	goat	Life Technologies

Antibodies were used in the dilutions indicated. Secondary antibodies were preabsorbed in *Wolbachia* uninfected embryos. DSHB, *Drosophila* Studies Hybridoma Bank.

Table 2.4 *In situ* hybridization oligonucleotides

Probe	Sequence
Wpan16S887	5'-ATCTTGCGACCGTAGTCC-3'
Wpan16S450	5'-CTTCTGTGAGTACCGTCATTATC -3'

Probes are labeled on the 5' end with Cy3 or Cy5 and were purchased from Integrated DNA Technologies. Probes were designed against conserved regions of the 16S gene and work for a wide variety of *Wolbachia* strains. Probe sequences were adapted from (Heddi *et al.*, 1999; Moreira *et al.*, 2009).

Table 2.5: Polymorphic markers for *wMel*

Genotype group	Genotype	IS5 WD1310 locus	IS5 WD0516/17 locus	Number of VNTR-141 motifs	Number of VNTR-105 motifs	Inversion
CS	<i>wMelCS</i>	yes	no	6	4	forward
	<i>wMelCS2</i>	yes	no	6	5	forward
MEL	<i>wMel</i>	no	yes	7	5	reverse
	<i>wMel2</i>	no	yes	6	5	reverse

Different subtypes of *wMel/CS Wolbachia* strains were determined according to (Ilinsky, 2013) and (Riegler *et al.*, 2005). Diagnostic PCR primers used for this analysis are listed in Table 2.6.

Table 2.6: PCR Primers

Gene	Forward Primer	Reverse Primer	Source
<i>For wMelPop/CS subtyping (octomom repeats):</i>			
Rpl32	CCGCTTCAAGGGACAGTATC	CAATCTCCTTGCGCTTCTTG	1
wsp	CATTGGTGTGGTGTGGTG	ACCGAAATAACGAGCTCCAG	1
WD0505	TGTTCTGGTGGATCATCTG	ACGCGAGCATCTCCATAAG	1
WD0506/WD0515	TTTGGCTCTTCTCCCTCTC	ATCAAGGCACACCACAAGGT	1
WD0507	GCATGACAGGGAAGAAGCTC	CTTTGCAGCTTCCTTTAGGC	1
WD0508	TCTAGCTTGCGGACAAGAAG	CTGCCTTCCACTTTCTTCC	1
WD0509	CCGTATAGCAGCAGGAGAGG	AGTGGCATGCCTCATAAGTG	1
WD0510	CCACTTGTGATCCATCCTG	GGCAGCCGTGGTAATGTATG	1
WD0511	CTTGGCTGCTATTACGATG	CGAAGCCCTGGTCTTAGTG	1
WD0512	ATGCTGCTAATTGGGACTGG	AGGCAATCGACCATACTTGC	1
WD0513	TTAACCGGCCAGTCTTATCG	AGCATGTCCTCTCTGCCATC	1
WD0514	CTGTGCTGAGAATCAAGAGG	CCTTCAAGCGAGGAGATTTG	1
WD0519	TGCAAGAAGAGAAAATCAAATAAGAG	TCCCTTGTAAGCGTTCTTTC	1
<i>For wMel subtyping:</i>			
VNTR 141	GGAGTATTATTGATATGCG	GAATAAGGTTAGTTGCAT	2
VNTR 105	GCAATTGAAAATGTGGTGC	ATGACACCTTACTTAACCGTC	2
IS5-WD0516-17	CCATCAAGGTCTCTTCA	TGCAAGGAAAATAAACCAG	3
IS5-WD1310	AGGAGAAGTGGTCTACGC	TGTTGCTGAGCTTTGCT	3

Sources for primers: 1. (Chrostek *et al.*, 2013), 2. (Riegler *et al.*, 2005), and 3. (Riegler *et al.*, 2012).

CHAPTER 3

Wolbachia Stem Cell Niche Tropism of the Ovary Across the *Drosophila* genus

Portions of this chapter were previously published in (Toomey *et al.*, 2013).

3.1 Introduction

The most common maternally transmitted bacteria in invertebrates are alphaproteobacteria belonging to the genus *Wolbachia*, representing the largest pandemic on the planet (reviewed by Werren *et al.*, 2008). These Rickettsia-like bacteria are estimated to infect a great number of invertebrate species, including insect vectors of infectious diseases and pathogenic filarial worms. Recently, it has been shown that *Wolbachia* strains derived from *D. mel*, when introduced into mosquito vectors, can invade and sustain themselves in mosquito populations (Walker *et al.*, 2011). Several phenotypes observed in *Drosophila* are also maintained in the mosquito non-native hosts: reduction of adult lifespan, reproductive manipulation, and resistance against several pathogens, including Dengue, Chikungunya, West Nile Virus, and both chicken and human Plasmodium (Moreira *et al.*, 2009; Kambris *et al.*, 2010; Hughes *et al.*, 2011; Walker *et al.*, 2011).

Because *Wolbachia* are maternally transmitted, their presence in the germline is essential for their vertical propagation to the next generation. However, *Wolbachia* are often found in several somatic tissues as well, and this distribution varies amongst different *Wolbachia* – host associations (Min *et al.*, 1997; Dobson *et al.*, 1999; Cheng *et al.*, 2000; McGraw *et al.*, 2004; Landmann *et al.*, 2010). The role of these bacteria in somatic cells is not clear.

Wolbachia can also move horizontally within and between species (Werren *et al.*, 1995; Huigens *et al.*, 2000; Cordaux *et al.*, 2001; Baldo *et al.*, 2008; Raychoudhury *et al.*, 2009). The mechanism by which horizontal transmission occurs in nature is poorly understood. Regardless of how *Wolbachia* reach a new host, after the initial infection event, reaching the germline is an essential requirement for successful transmission to the next generation (Werren *et al.*, 2008). It has been previously reported in *D. melanogaster* (*D. mel*) that upon recent infection through microinjection, *Wolbachia* enter the region of the ovary containing the germarium. Several germaria reside at the anterior tip of each ovary and house all the stem cells necessary to make an egg (Figure 1.2, Figure 3.1A). Within the germarium, the major route for *Wolbachia* to enter the germline in this artificial infection model is through the somatic stem cell niche (SSCN, Figure 3.1A, light blue cells) (Frydman *et al.*, 2006). The SSCN is the microenvironment that harbors the somatic stem cell (Figure 3.1A, dark blue cells), which in turn generates the somatically derived follicle cells that envelope the germline and secrete the eggshell. This observation in *D. mel* raised the possibility of tropism for stem cell niches as a mechanism to facilitate reaching the germline during horizontal infection.

The same work also showed that *Wolbachia* accumulate at the SSCN in maternally infected flies (Frydman *et al.*, 2006). Additionally, in another species of fruit fly, *D. mauritiana*, *Wolbachia* also target the germline stem cell niche (GSCN, Figure 3.1A, green cells) in long-term maternally infected flies (Fast *et al.*, 2011). The GSCN is a somatic structure at the anterior tip of the germarium, composed of terminal filament and cap cells (Figure 3.1A; TF, light green; CC, dark green) that support the germline stem cells (GSC,

Figure 3.1A, yellow cells). The GSCs are the source of the germline cells that develop into the eggs. These observations and subsequent work in other invertebrates (Hosokawa *et al.*, 2010; Sacchi *et al.*, 2010; Landmann *et al.*, 2012) suggest that stem cell niche tropism plays a widespread role in germline infection during long-term maternal transmission of *Wolbachia*, in addition to a potential role during horizontal transmission.

Here, utilizing cell biological, phylogenetic, genetic and transinfection tools, we provide evidence that stem cell niche tropism is an evolutionarily conserved mechanism for *Wolbachia* hereditary and non-hereditary transmission. Our data revealed two patterns of niche tropism in the ovary among naturally infected *Drosophila* species: 1) somatic stem cell niche tropism only; and 2) somatic and germline stem cell niche tropism. Beyond the qualitative differences of targeting different niches, bacterial density, distribution, and frequency of stem cell niche infection varies according to *Drosophila-Wolbachia* pairs. Using ecologically diverse *Drosophila-Wolbachia* pairs we show that this tropism is a widespread occurrence across the *Drosophila* genus. Phylogenetic analyses reveal selective pressures promoting strong conservation of the same pattern of niche tropism amongst closely related *Wolbachia* strains. Furthermore, quantification of bacterial densities across different regions of the germarium shows an increase of *Wolbachia* loads in the germline during or immediately after interaction with infected stem cell niches, supporting previous suggestions of a contribution of *Wolbachia* from stem cell niches towards vertical transmission. Finally, through hybrid crosses and transinfection experiments, we show for the first time that *Wolbachia* encoded factors, rather than the host genetic background, are the major determinants of different patterns of stem cell niche

tropism. These results support the role of stem cell niches as a key component for spreading of *Wolbachia* in the *Drosophila* genus and provide mechanistic insights into this unique tissue tropism.

3.2 *Wolbachia* tropism to the somatic stem cell niche is pervasive across the *Drosophila* genus in all species tested

To determine whether niche targeting is an evolutionarily conserved occurrence across the *Drosophila* genus, we conducted a survey of eleven different *Wolbachia* strains that naturally infect nine different *Drosophila* species (Table 2.1). Of the nine species comprising the *D. mel* subgroup, seven are naturally infected with *Wolbachia*. We analyzed all of them except for *D. santomea*. The publicly available *D. santomea* stock that we obtained was not infected (Mateos *et al.*, 2006). However, we characterized niche tropism in natively infected *D. yakuba* and *D. teisseri* flies that are closely related to *D. santomea*, together comprising the *yakuba* complex. The *Wolbachia* strains that infect the *yakuba* host complex are also closely related, being described as identical in some papers (Zabalou *et al.*, 2004). Therefore, all the major *Wolbachia* strains infecting the *D. mel* subgroup are present in this study. In addition three other species representative of major groups across the *Drosophila* genus (naturally infected with *Wolbachia*) were analyzed (*D. innubila*, *D. tropicalis*, and *D. ananassae*). Using immunohistochemistry, we quantified the frequency of *Wolbachia*'s niche tropism in the germaria of all eleven *Wolbachia* strain-*Drosophila* species pairs. In every ovary analyzed, we found that *Wolbachia* preferentially infect the border region (BR) between regions 2a and 2b of the germarium (Figure 3.1A, for controls see Figure 2.1). This region contains the somatic stem cell niche (SSCN) and preferential

Wolbachia infection at the BR characterizes SSCN tropism. By comparing *Wolbachia* levels at the BR to the neighboring somatic regions 2a and 2b, we found that *Wolbachia* was enriched in the SSCN in 100% of individuals for each species (N=119 flies, Figure 3.1 B-L). Visual assessment of confocal imaging of approximately 10 randomly sampled germaria from each ovary showed a frequency of SSCN tropism of greater than 80% (N=1194 total germaria, Figure 3.1M, P=0.0012, Table 3.1). To quantify levels of *Wolbachia* enrichment at the SSCN, representative confocal Z stacks were subjected to image analysis of *Wolbachia* voxel density in the soma of the different germarial regions (Figure 3.2, Tables 3.2 and 3.3). In every species analyzed, there was an increase in *Wolbachia* load in the soma of the SSCN region normalized to the somatic cells in adjacent region 2b ranging from 2 to 59-fold (Figure 3.3, T-test between BR and 2b statistically significant, P<0.01 for all species). This analysis indicates a strong selective pressure for an evolutionarily conserved *Wolbachia* tropism to the SSCN.

3.3 *Wolbachia* target the germline stem cell niche in a subset of species

In addition to *Wolbachia* tropism to the SSCN, we observed *Wolbachia* infection in the cap cells and terminal filament cells comprising the germline stem cell niche (GSCN) (Figure 3.1A, green TF and CC). Infection of the CC vs. the TF cells was fairly similar, and when correlated, have an $R^2=0.97$ (Figure 3.4, $P=6.6 \times 10^{-9}$). Since the frequency of infection is similar between the two cell types, the analysis shown of GSCN tropism refers to an average between infection of the TF cells and the CCs. Six of eleven *Drosophila-Wolbachia* pairs analyzed showed GSCN tropism (Figure 3.1 G-L). Occurrence of GSCN tropism is more variable than SSCN tropism, with frequencies ranging from 37% to 99%

of GSCNs targeted (Figure 3.1N, Table 3.1, N=647 total germaria). ANOVA analyses defined three distinct groups: high frequency (HF) of GSCN targeting (Figure 3.1 J-L; Figure 3.1N, P=0.80), moderate frequency (MF) of GSCN targeting (Figure 3.1 G-I; Figure 3.1N, P=0.087), and low/no frequency (LF) of GSCN targeting (Figure 1 B-F; Figure 1N, P=0.44). In species with GSCN tropism, voxel intensity measurements showed that *Wolbachia* density is from 2.5 to 26.5-fold enriched in the GSCN normalized to region 2b soma (Figure 3.5, Table 3.4). Relative to SSCN tropism, targeting of the GSCN occurred at a lower frequency and density. These observations show that although targeting of stem cell niches in the *Drosophila* ovary is a widespread occurrence, the patterns of distribution are not the same in all *Drosophila* host-*Wolbachia* strain pairs.

3.4 *Wolbachia* also target the escort cells

In region 1 of the germarium, in addition to tropism to the GSCN, we also observed high levels of *Wolbachia* in the escort cells (Figure 3.6). The escort cells are a stable, non-dividing, stromal population of cells that are attached to the basement membrane of the germarium and support the progression of early germline cysts in region 1 and 2A of the germarium (Figure 3.1A)(Morris *et al.*, 2011). Because the Vasa antibody staining did not consistently allow clear visualization of escort cells in all species, this analysis was not possible across the genus, and was restricted to *D. mauritiana*. We found that approximately 50% of the escort cells analyzed in *D. mauritiana* were highly infected with *Wolbachia* relative to the surrounding germline (Figure 3.6D), indicating that there may be an additional tropism to the escort cell population promoting somatic routes for germline infection.

3.5 Phylogenetic analyses suggest that differential niche tropisms are mediated by *Wolbachia* encoded factors

In broad terms, we see two different patterns of stem cell niche tropism in the *Drosophila* ovary: 1) targeting of only the SSCN (herein referred to as SSCN pattern) or 2) targeting of both the SSCN and the GSCN (herein referred to as GSCN pattern). This observation of differential patterning of stem cell niches led us to investigate the relative contributions of host factors and bacterial factors toward the distinct *Wolbachia* tropism patterns. We reconstructed the evolution of niche tropism on phylogenetic trees of both *Wolbachia* and *Drosophila* (Figure 3.7) (Jeffs *et al.*, 1994; Paraskevopoulos *et al.*, 2006) to determine if patterns of niche tropism were primarily determined by factors derived from the *Wolbachia* strains or derived from the *Drosophila* host species. To quantify the correlation of niche tropism pattern to the two different phylogenies, we utilized a computer simulation model of randomized character distributions to compare with the distribution of niche tropism pattern on each of the phylogenies (Figure 3.8) (Maddison *et al.*, 2005). Observed niche tropism correlated with the *Drosophila* phylogenetic tree has a tree length of 4 and out of 1000 random character distributions, 80.8% require 4 or fewer steps (Figure 3.8 A and B). Conversely, observed niche tropism correlated with the *Wolbachia* phylogeny requires 3 steps (Tree length = 3) and out of 1000 computer simulated random characters, only 8.7% require 3 or fewer steps (Figure 3.8 C and D) (Maddison *et al.*, 2005). There is an approximately 10-fold lower probability that the association of niche tropism with the *Wolbachia* phylogeny is due to random chance than the association with the *Drosophila* phylogeny. Therefore, closely related *Wolbachia*

strains are more likely to display similar patterns of tropism compared to the tropism patterns observed in closely related *Drosophila* species. This suggests that the different patterns of niche tropism evolved in *Wolbachia* and that the pattern of shared *Wolbachia* niche tropism in *Drosophila* results from characteristics of the infecting *Wolbachia* strain rather than characteristics of the host *Drosophila* species.

3.6 Hybrid crosses confirm that bacterial factors mediate stem cell niche tropism

The phylogenetic analyses suggest that *Wolbachia* factors mediate differential stem cell niche tropism patterns. To experimentally evaluate this hypothesis, we generated hybrid flies between *Drosophila* species harboring two different *Wolbachia* strains that display the two different *Wolbachia* tropism patterns, using genetic introgression (Figure 2.1A). The rationale for this experiment is: if the pattern of tropism is mediated by the *Wolbachia* strain, the *Wolbachia* patterning in the germaria in the hybrid host will be the same as the original maternal host, regardless of the introgressed male host genetic background (Figure 2.1B).

Hybrid fly lines were created by crossing *D. mauritiana* flies infected with *Wolbachia* *w*Mau, which display a GSCN tropism pattern, and *D. sechellia* flies infected with *Wolbachia* *w*Sh, which display a SSCN tropism pattern. The corresponding hybrid flies' genital arches matched the appropriate genetic background, as indicated by the blue arrows in Figure 3.9, demonstrating a successful introgression of most of the paternal genome into the F₅ hybrid. *Wolbachia* *w*Mau infecting both the parental *D. mauritiana* and hybrid *D. sechellia*, display a high frequency of GSCN tropism pattern (greater than 85%, Figure 3.9 A and B green bars, Table 3.5, N=209 total germaria). In contrast, *Wolbachia*

wSh, infecting both the parental *D. sechellia* and hybrid *D. mauritiana* displays high frequencies of the SSCN tropism pattern, with greater than 90% of germaria analyzed only infecting the SSCN (Figure 3.9 A and B red bars, Table 3.5, N=260 total germaria). Regardless of genetic background, both *Wolbachia* strains display the same niche tropism patterns in both the maternal and hybrid hosts. Logistic regression analysis was performed to evaluate the relative contributions of the *Wolbachia* strain and the host genetic background to the differential patterns of stem cell niche tropism. We found no evidence of host influence on niche tropism pattern (P=0.18); however, the *Wolbachia* strain does have a highly statistically significant effect (P=4.7x10⁻²²). Analysis of *Wolbachia* density in representative images confirms GSCN tropism in *wMau* infected flies and SSCN tropism in *wSh* infected flies (Figure 3.9C, Table 3.6).

These results are in agreement with our phylogenetic analysis and support the hypothesis that stem cell niche tropism is largely mediated by *Wolbachia* factors rather than the host genetic background.

3.7 Maternally inherited components have no influence on niche tropism pattern

During the hybrid crosses, together with the *Wolbachia* strain, other maternally inherited components, such as the mitochondria, are also transmitted. To eliminate the possibility that maternally transmitted organelles and other factors have a role in determining the previously tested differences in *Wolbachia* niche tropism, we utilized a fly line whose *Wolbachia* infection was established via microinjection. This line was previously generated by *Wolbachia* isolation from one host species followed by injection into another species (Poinsot *et al.*, 1998). Niche tropism of *D. simulans* flies trans-infected

with *wMel* via embryonic microinjection was assessed. The results indicate that the *Wolbachia* strain is necessary and sufficient to determine the pattern of niche tropism in a non-native host. *wMel* infected flies always display *Wolbachia* infection in the SSCN only, regardless of genetic background and maternally inherited components (Figure 3.10 A and B, Table 3.7, N=246 total germaria). Logistic regression analysis confirms that the *Wolbachia* strain has a significantly greater effect on niche tropism pattern than the host genetic background ($P= 6.7 \times 10^{-7}$ and $P=0.76$, respectively). Analysis of *Wolbachia* pixel density of representative images supports niche tropism quantification, showing high *Wolbachia* densities only in the SSCN of *wMel*-infected flies (Figure 3.10C, Table 3.8).

3.8 *Wolbachia* factors direct qualitative differences within niche tropism pattern

We also observed variability in the pattern of *Wolbachia* distribution in the TF cells. Some TFs were fully infected, with all cells densely infected with *Wolbachia*; others had a discontinuous pattern of infection, with only some TF cells densely infected, interspersed with non-infected TF cells. Interestingly, two *Wolbachia* strains that naturally infect *D. simulans* had this noticeable difference, which was most evident in young flies. *Wolbachia wRi* displays a discontinuous TF pattern of infection (Figure 3.1H and Figure 3.11A); *Wolbachia wNo*, fully infects the TF (Figure 3.1J and Figure 3.11A).

Since we have shown that *Wolbachia* factors mediate the overall patterns of niche tropism, we investigated whether they also influence qualitative differences within the same pattern. After backcrossing to introgress the host genetic backgrounds (Figure 2.1), we observed that *wRi* infected flies, regardless of host strain genetic background, display a high frequency of discontinuous terminal filament infection, with approximately 80% of

highly infected niches having a discontinuous pattern (Figure 3.11, Table 3.9, N=230 total germaria). *Wolbachia* wNo infected flies display a low frequency of discontinuous terminal filament infection, with approximately 20% of infected niches having a discontinuous pattern, regardless of host strain genetic background (Figure 3.11, Table 3.9, N=242 total germaria). Logistic regression analysis confirms that the *Wolbachia* strain plays a more significant role in the discontinuous GSCN pattern than the fly genetic background ($P=6.5 \times 10^{-11}$ and $P=0.54$, respectively). These results demonstrate that *Wolbachia* encoded factors also direct specific differences in the distribution of bacteria within the GSCN.

3.9 *Wolbachia* levels in the germline increase with proximity to infected niches

To assess the contribution of stem cell niche tropism towards *Wolbachia* enrichment in the germline, we quantified the *Wolbachia* density in the germline in the different germarial regions of each of the *Drosophila-Wolbachia* pairs (Figure 3.12, Table 3.10). For contribution from the SSCN, we compared the density of *Wolbachia* in germline cysts in region 2a to the density of *Wolbachia* in germline cysts in region 2b. These two regions contain germline cells before (2a) and after (2b) developing cysts pass through the niche. In all species, except *D. tropicalis*, we observed a similar trend: after passage through the border region containing the highly infected SSCNs, the levels of *Wolbachia* in germline cysts in region 2b are higher than the levels of *Wolbachia* in region 2a, with fold-changes (2b/2a) ranging from 1.3 to 25 (Figure 3.13 A-L). Although there is high variability in *Wolbachia* load from germline cyst to germline cyst, 7 of 11 species, have a statistically significant increase of *Wolbachia* load from 2a to 2b (see white arrows Figure 3.13 B-F, J, K; Quantification, M, T-test: $P < 0.05$, Table 3.11).

For contribution from the GSCN, we compared the relative fraction of *Wolbachia* in region 1 of the germline across species with GSCN tropism and without GSCN tropism (Figure 3.12N). Species with GSCN tropism had a higher relative density of *Wolbachia* in region 1 (as compared to the whole germarium) than species with only SSCN (green asterisks in Figure 3.13 G-L; Quantification, N). In the majority of *Drosophila* species analyzed, *Wolbachia* tropism to the stem cell niches correlates with higher densities of *Wolbachia* in the adjacent germline. These results agree with previous studies (Frydman *et al.*, 2006; Sacchi *et al.*, 2010), supporting a passage of *Wolbachia* from the niche into the germline.

3.10 The increase of *Wolbachia* density from regions 2a to 2b is contributed to by *Wolbachia* proliferation in the germline and niche

For the niche to be a source for *Wolbachia* into the germline, we expect *Wolbachia* to be dividing in the niche. Using an antibody against FtsZ, we observed substantial *Wolbachia* division within the SSCN (Figure 3.14A). In addition to passage from the SSCN, *Wolbachia* actively divide in the germline, which also contributes to the increase in *Wolbachia*'s density in region 2b (Figure 3.14 B and C). Region specific differences in the rate of *Wolbachia* division could play a major role in the increase of *Wolbachia* in region 2b. However, our analysis indicates that the fraction of *Wolbachia* dividing in both regions 2a and 2b of the germarium are the same (Figure 3.14 B and C). Even with the same division rate of *Wolbachia* in these regions, differences in cyst development timing could also play a role in the increase of *Wolbachia* density in region 2b. However, studies in *D. mel* demonstrate that the developmental time that germline cysts remain in region 2b is not

significantly different than the time the germline cysts are present in the surrounding regions 2a and 3, ruling out this possibility in at least *D. mel* (Drummond-Barbosa *et al.*, 2001). These data suggest that *Wolbachia* division within the germline, in combination with *Wolbachia* passage from the niche, contribute to the increase of *Wolbachia* density in region 2b.

3.11 Discussion

To understand the spread of *Wolbachia* in nature, it is important to elucidate the mechanisms of horizontal and vertical transmission. Because the majority of transmission events are maternal, to effectively infect a population *Wolbachia* must infect the female's germline during both long-term stable vertical transmission and recent horizontal introduction into a new host. Here we provide evolutionary, cytological, genetic, and developmental evidence for a mechanism in which stem cell niche tropism promotes germline colonization across the *Drosophila* genus. We also demonstrate that factors encoded by the *Wolbachia* strain, rather than the host species, are the major determinants of the type of stem cell niche that is infected.

In a survey of niche tropism, we show that *Wolbachia* display tropism for two different stem cell niches in the *Drosophila* ovary – the SSCN and the GSCN. Several studies have described *Wolbachia* preferential infection of different tissues, host cells, and sub-cellular locations in the *Drosophila* genus, including adult brain, embryonic neuroblasts, specific regions of the oocyte during oogenesis, and posterior or anterior areas of the early embryo (Min *et al.*, 1997; Veneti *et al.*, 2004; Serbus *et al.*, 2007; Albertson *et al.*, 2009). Considering *Wolbachia*'s transmission across generations, a site in the host of

particular interest is the germlasm, which is a highly specialized, maternally synthesized cytoplasm that is deposited in the posterior pole of the egg and induces the formation of the germline in the embryo (Illmensee *et al.*, 1974; reviewed by Santos *et al.*, 2004). During late oogenesis and early embryonic development, *Wolbachia* efficiently colonize the germlasm in *D. mel* giving rise to a highly infected germline, ensuring *Wolbachia* transmission to the subsequent generation (Hadfield *et al.*, 1999; Veneti *et al.*, 2004). However, germlasm infection is not observed in several other *Drosophila* species (Veneti *et al.*, 2004; Serbus *et al.*, 2007). Surprisingly, targeting of the SSCN is more prevalent in the *Drosophila* genus than targeting of the germlasm. To our knowledge, with the exception of infection of the adult oocyte, the preferential infection of the SSCN reported here is the most conserved *Wolbachia* tropism reported in the *Drosophila* genus.

Given that *Wolbachia* does not colonize the germlasm of the embryo in every *Drosophila* species, there must be an alternative mechanism to ensure its vertical transmission. The strong phylogenetic conservation of patterns and the pervasive presence of tropism for stem cell niches in the *Drosophila* germarium are suggestive of a significant role for niche tropism in transmission. Previous work has implicated stem cell niche tropism as a mechanism facilitating horizontal transmission of *Wolbachia* in *D. mel* (Frydman *et al.*, 2006). Our confocal imaging analysis suggests that stem cell niches in the *Drosophila* germarium also play a role in vertical transmission of *Wolbachia*. Similar to our findings, there is a surprising observation from the *Wolbachia* strains infecting filarial nematodes. In the filarial worm, *Wolbachia* are excluded from the precursor of the germ cell lineage; infection of the gonad happens later in development, through the invasion via

the distal tip cell, the nematode equivalent of the stem cell niche (Landmann *et al.*, 2012). Furthermore, studies on a bedbug and a leafhopper suggest that *Wolbachia* are transmitted to the germline via a putative stem cell niche (Hosokawa *et al.*, 2010; Sacchi *et al.*, 2010). These observations support a hypothesis of stem cell niche tropism as a mechanism for *Wolbachia* dissemination shared during both horizontal and vertical transmission.

Our data clearly shows that the SSCN prevails over the GSCN in terms of occurrence and evolutionary conservation. To provide an explanation for these observations, we propose a model that considers *Wolbachia* transmission to the germline during development from the stem cell niches. The differences in the anatomic features between niches and associated cells as well as the developmental time periods in which *Wolbachia* can be transmitted from each niche suggest that the SSCN is better suited for *Wolbachia* transmission to the germline.

The model presented in Figure 3.15 displays potential routes of *Wolbachia* entry into the germline from the surrounding niches and other somatic cells during *Drosophila* oogenesis. The GSCN contacts the germline stem cell, providing a potential route for the *Wolbachia* present in this niche to enter the germline (Figure 3.15C, dark blue arrows). In addition, when escort cells are highly infected, it is possible to have transmission from these somatic cells into the germline until the developing cyst reaches the border region (BR) (Figure 3.15C, light blue arrow). Therefore, transmission into the germline could occur for a total of approximately 2.5 days, the estimated time for germline transit from the germline stem cell niche to the BR (Figure 3.15B, see blue line in timeline) (Drummond-Barbosa *et al.*, 2001; Morris *et al.*, 2011).

In comparison, the SSCN provides several routes for *Wolbachia* transmission into the germline (Figure 3.15 D-G), both direct and indirect. Because the SSCN contacts all developing germline cysts, it can transmit *Wolbachia* directly into the germline cells that must pass through the border region (Figure 3.15 B and D, red arrows). This was initially suggested to be the case for *D. mel* by confocal analysis (see Sup Table 1 in ref. Frydman *et al.*, 2006), further corroborated by EM studies (Sacchi *et al.*, 2010). The data presented here suggest that the SSCN can deliver *Wolbachia* directly into the germline in all species of *Drosophila* analyzed in this study.

The SSCN can also transmit *Wolbachia* indirectly. The infected niche is a constant source of *Wolbachia* into the SSC, which, in turn, divides and transmits *Wolbachia* into the developing follicle cells (Figure 3.15D, orange arrows) (see also Supplementary Fig. 2b–d and Supplementary Movie in reference Frydman *et al.*, 2006). The follicle cells can transmit *Wolbachia* into the germline of developing egg chambers through the remaining stages of germline development, providing an extended period of developmental time for transmission (Figure 3.14B, developmental stages indicated by orange line; Figure 3.15 E-G, orange arrows). Furthermore, several yolk proteins produced by the follicle cells are actively transported into the oocyte during the final stages of oogenesis (Brennan *et al.*, 1982). This process may provide a facilitated mechanism for *Wolbachia* present in the follicle cells to transfer into the oocyte (Figure 3.15G) and indeed we do see *Wolbachia* at the follicle cell-oocyte interface in stage 8 egg chambers by electron microscopy (Figure 3.16). From the border region, it takes approximately five days for the completion of oogenesis (He *et al.*, 2011). Compared to the previous 2.5 days of cyst development in

regions 1 and 2A where there is the potential for *Wolbachia* transmission from the GSCN and escort cells, the developmental time available for transmission of *Wolbachia* derived from the SSCN is about twice as long (Figure 3.15 A and B, blue line vs. red/orange line in timeline). Ultimately, it is easier for *Wolbachia* to reach the germline through the SSCN (rather than the GSCN) during vertical transmission and probably during horizontal transmission as well. These developmental and anatomical features of the niches provide an explanation for the phylogenetic, genetic and cytological data presented here.

This work highlights bacterial localization as a fundamental aspect of *Wolbachia*-host interactions being maintained during *Wolbachia* evolution. Our current understanding of the mechanisms involved in *Wolbachia* localization is limited (Serbus *et al.*, 2008). Towards dissecting the mechanistic basis of stem cell niche tropism, we investigated the relative role of bacterial versus host factors in the different patterns of niche tropism. Through hybrid crosses and transinfection experiments, we showed that bacterial intrinsic factors are the major determinant of the pattern of niche tropism and also determine differences within the same pattern.

There are extensive comparative genomic analyses of different *Wolbachia* strains utilized in this study (Klasson *et al.*, 2009; Baldo *et al.*, 2010; Siozios *et al.*, 2013). At this point, we cannot attribute differences in the targeting of stem cell niches to specific genes or proteins due to a large number of genomic differences across the *Wolbachia* strains analyzed (Baldo *et al.*, 2010; Siozios *et al.*, 2013). Indeed, it has been suggested that *Wolbachia* is one of the most highly recombining intracellular bacterial genomes known to date (Klasson *et al.*, 2009). Nevertheless, the data presented here provide the foundation

for future approaches towards the identification of genetic pathways mediating *Wolbachia*'s stem cell niche tropism in hosts.

Wolbachia-based technologies are emerging as a promising tool for the control of vectors of deadly human diseases, including Dengue fever, West Nile Virus and malaria (Moreira *et al.*, 2009; Kambris *et al.*, 2010; Hoffmann *et al.*, 2011; Hughes *et al.*, 2011; Pan *et al.*, 2012). Understanding the basis of *Wolbachia* targeting of specific tissues in the host and its consequences towards bacterial transmission will provide further mechanistic insight into their extremely successful propagation and is also relevant for developing new *Wolbachia*-based vector control approaches.

Figure 3.1: *Wolbachia* tropism for stem cell niches is present across the *Drosophila* genus, with specific patterns of distribution

A. Representative diagram of a *Drosophila* germarium with the regions and cell types indicated: GSCN, in green [formed by TF cells (light green) and cap cells (dark green)]; GSC in yellow; escort cells in grey; SSCN in light blue; SSC in dark blue; and germline in red; BR indicates border region. **B-L.** *Wolbachia* distribution in germaria of different *Drosophila* species. DNA is in blue, germline marker (Vasa) is in red and *Wolbachia* is in green. *Wolbachia* highly infect the SSCN in all species and also infect the GSCN in several species (G-L). Scale Bar 10 μ m. **M.** Frequency of SSCN tropism. **N.** Frequency of GSCN tropism. Brackets indicate groups with statistically similar frequencies. Groups are statistically significantly different from each other. N \approx 100 each, for details see Table S2. Error bars represent SEM.

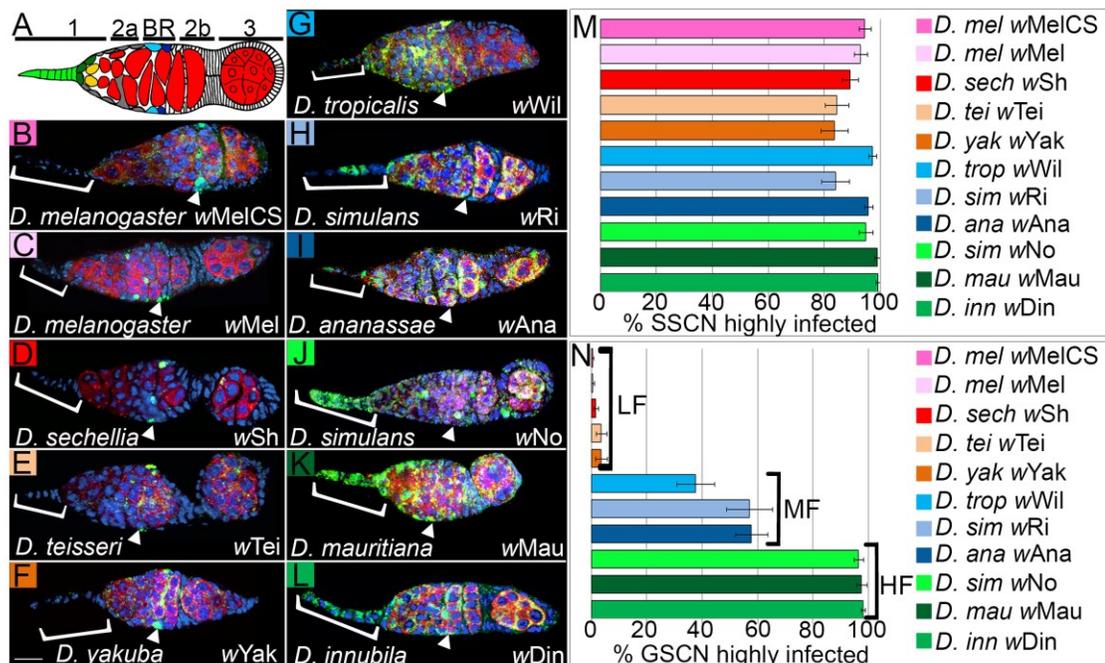


Figure 3.2: The border region has the highest fraction of *Wolbachia* in the soma per region of germarium

Representative images for each *Drosophila-Wolbachia* pair were analyzed using MatLab to measure the *Wolbachia* pixel density in each of the regions of the germaria as defined in the materials and methods (N=10 for each species). Error bars represent SEM. In every species analyzed, the fraction of *Wolbachia* in the soma is the highest in the border region, varying from 30% to 80% of total *Wolbachia* infecting somatic cells.

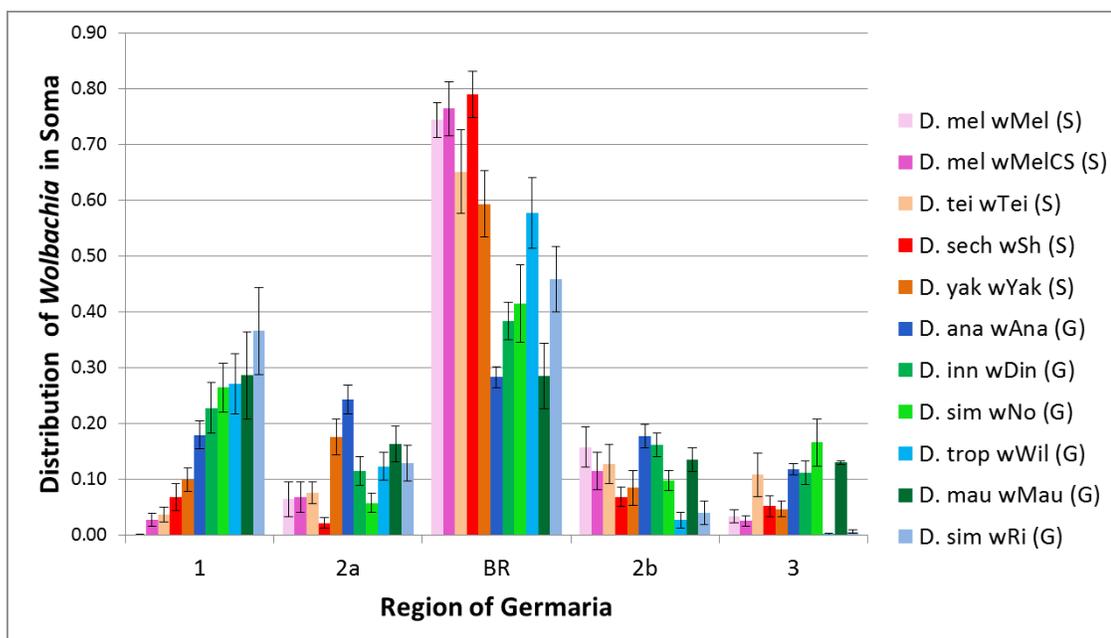


Figure 3.3: *Wolbachia* density in the soma of the border region is significantly higher than the adjacent somatic cells in region 2b

Representative images for each *Drosophila-Wolbachia* were analyzed using MatLab to measure the *Wolbachia* pixel density in each of the regions of the germaria as defined in Materials and Methods, Chapter 2 (N=10 for each species). *Wolbachia* density in the soma of the BR containing the SSCN is significantly higher than the adjacent somatic cells in region 2b. P-values represent that the differences in *Wolbachia* density between BR and 2b are statistically significantly different (T-test). Error bars represent SEM and **P<0.01, ***P<0.001, ****P<0.0001.

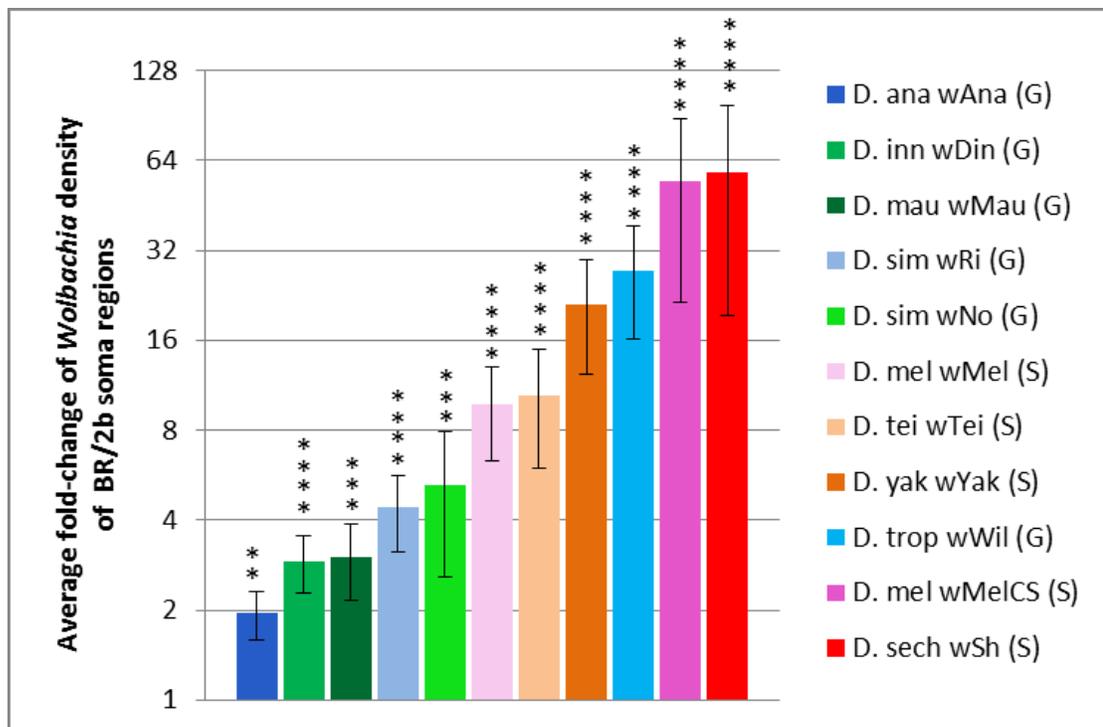


Figure 3.4: Correlation of tropism to the cap cells and terminal filament

Germline stem cell niche tropism consists of tropism to two main cell types comprising the GSCN: the cap cells (CC) and the terminal filament (TF) cells. Infection of the CC vs. the TF cells is fairly similar, and has an $R^2=0.97$ ($P=6.6 \times 10^{-9}$) ($N \approx 100$ germaria each, for details see Table S2).

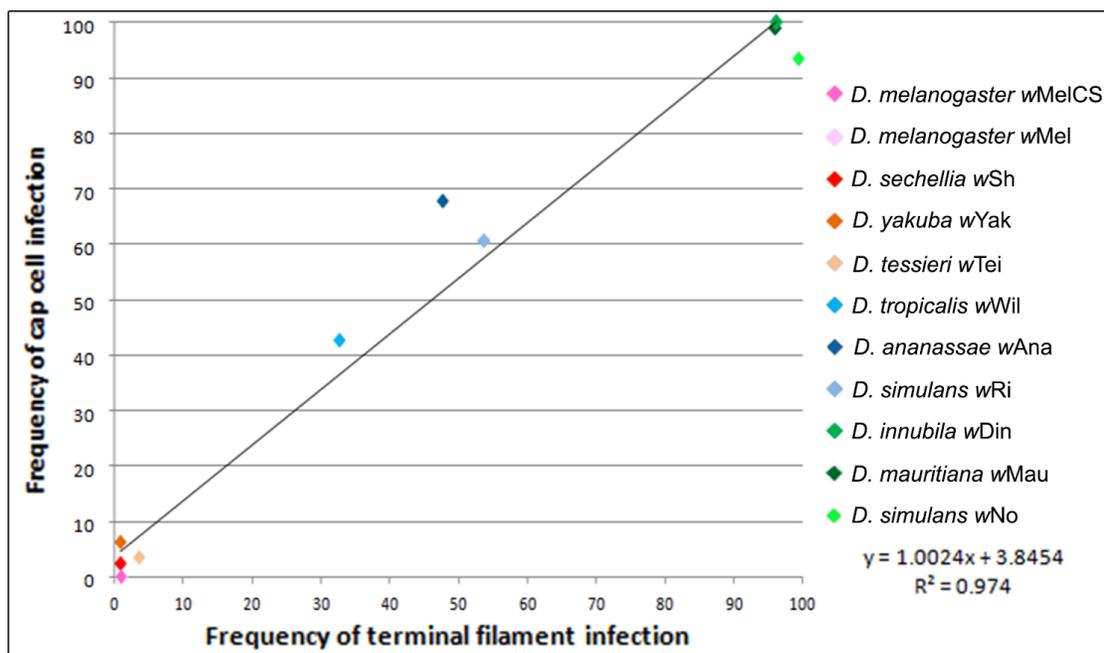


Figure 3.5: *Wolbachia* density in the GSCN (normalized to region 2b) is significantly higher in most species with GSCN tropism

Representative images for each *Drosophila-Wolbachia* pairs were analyzed using MatLab to measure the *Wolbachia* pixel density in each of the regions of the germaria as defined in the Material and Methods, Chapter 2 (N=10 for each species). *Wolbachia* density in the GSCN is statistically significantly higher in most species with GSCN tropism (T-test between GSCN and somatic region 2b). Error bars represent SEM and *P<0.05, **P<0.01, ***P<0.001.

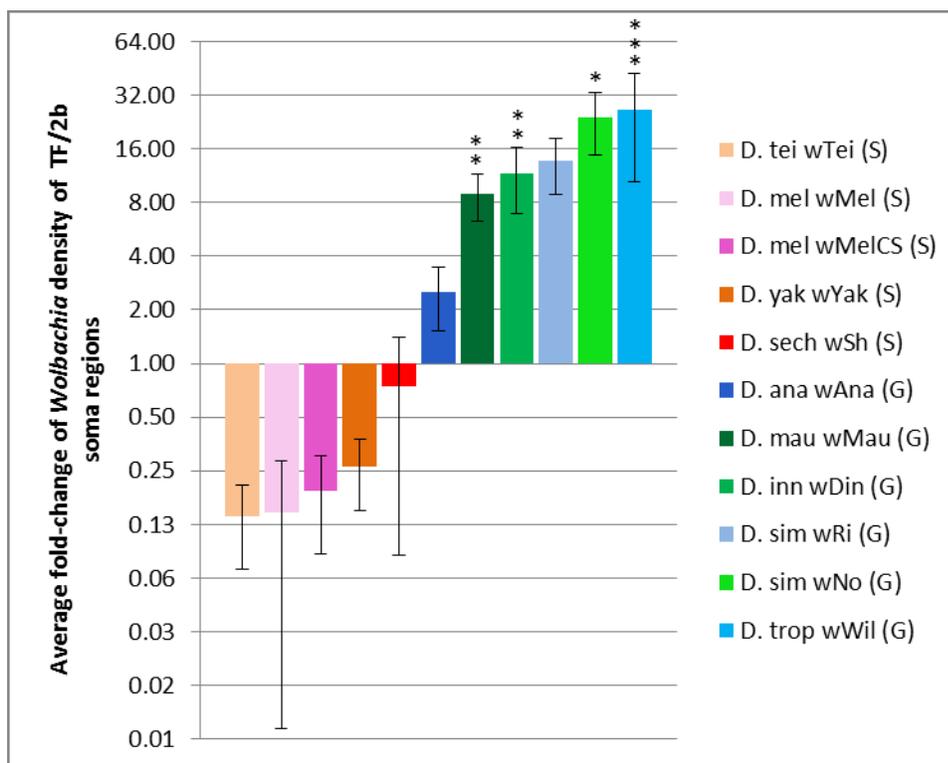


Figure 3.6: *Wolbachia* target the escort cells in *Drosophila mauritiana*

Yellow arrowhead indicates *Wolbachia* highly targeting an escort cell. **A.** Grey scale image of *Wolbachia* channel only. **B.** Grey scale image of Vasa channel only. **C.** Merge, showing *Wolbachia* highly infecting an escort cell. **D.** Quantification of *Wolbachia* tropism to escort cells (N=22). Error bar represents SEM, scale bar 10 μ m.

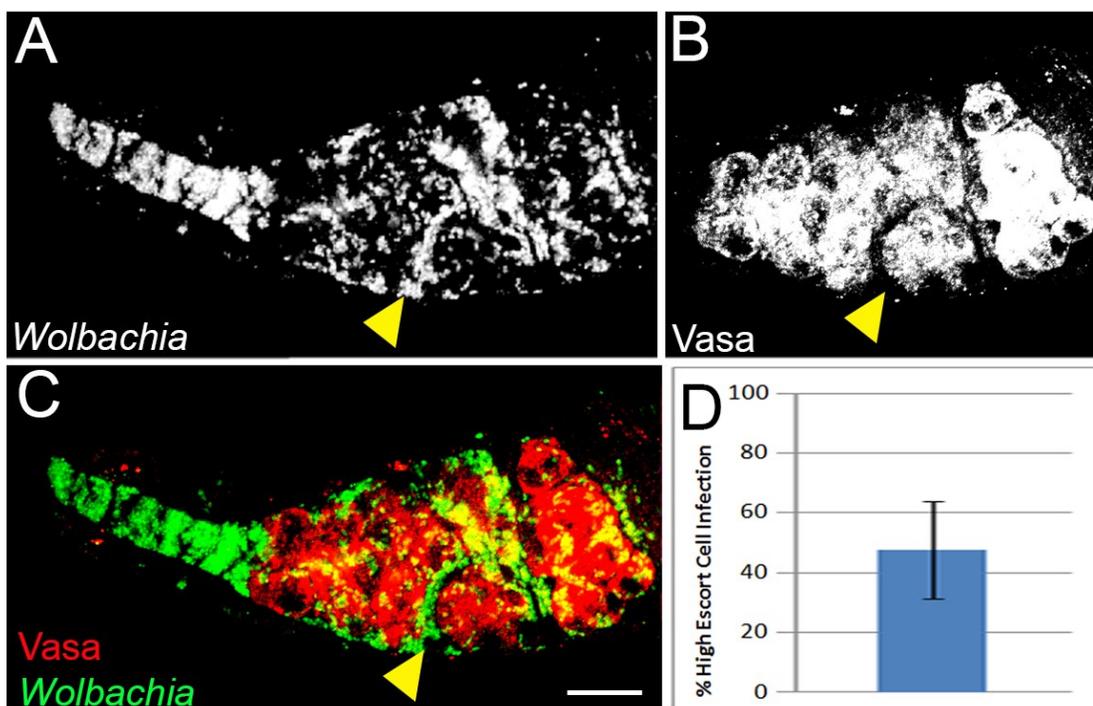


Figure 3.7: Correlation of stem cell niche targeting pattern with *Drosophila* and *Wolbachia* phylogenies

Different patterns of niche targeting are correlated with *Drosophila* and *Wolbachia* phylogenies (phylogenies adapted from Jeffs *et al.*, 1994; Paraskevopoulos *et al.*, 2006) (MYA= million years ago). Green, blue, and red lines indicate high, moderate, and low frequency of GSCN tropism, respectively.

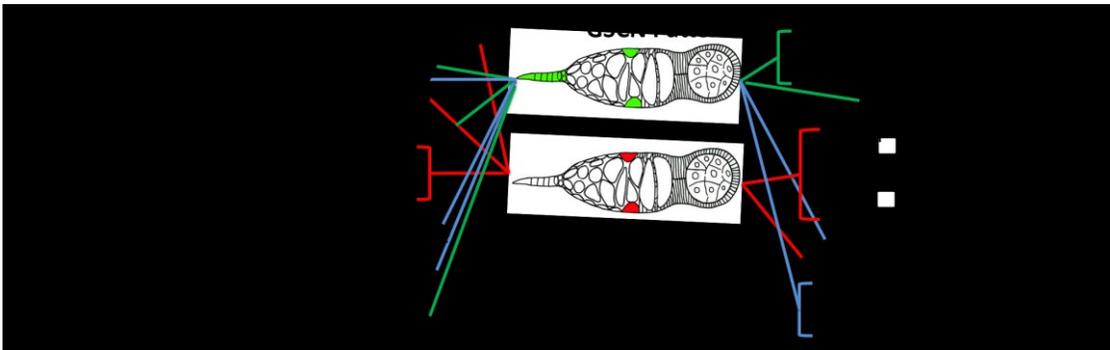


Figure 3.8: Random fit distribution of niche tropism on *Wolbachia* and *Drosophila* phylogenies

A. and C. GSCN tropism character is traced and character fit to the phylogenies was calculated using MacClade software (Maddison *et al.*, 2005). **A.** Stem cell niche tropism character fit to the *Drosophila* phylogeny. Phylogeny based on *alcohol dehydrogenase* gene (Jeffs *et al.*, 1994). **B. and D.** A set of 1000 random characters was evolved to assess the probability of the GSCN tropism character fit to the phylogeny due to chance. The probability of a fit as good, or better than the true character was calculated for each phylogeny. **B.** There is an 80.7% probability that the GSCN tropism character distribution on the *Drosophila* phylogeny is due to random chance. **C.** Stem cell niche tropism character fit to *Wolbachia* phylogeny. Circles represent nodes with a maximum likelihood boot strap value of less than 50. *Wolbachia* phylogeny based on multilocus sequence typing (Paraskevopoulos *et al.*, 2006). **D.** There is an 8.7% probability that the GSCN tropism character distribution on the *Wolbachia* phylogeny is due to random chance.

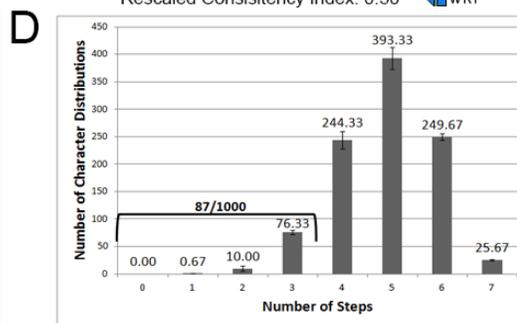
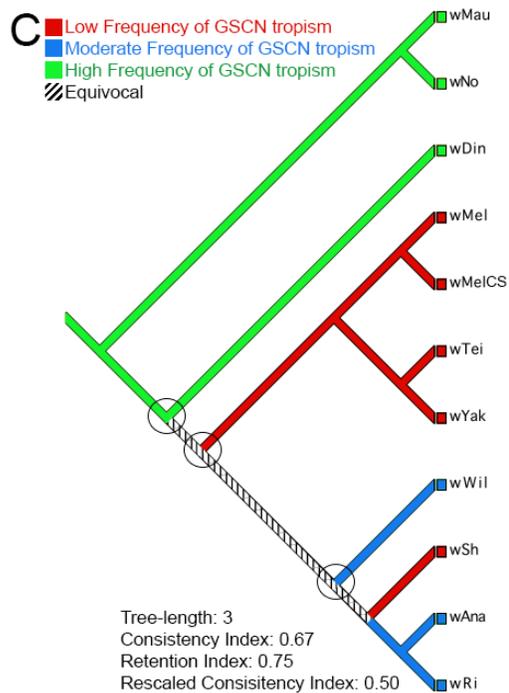
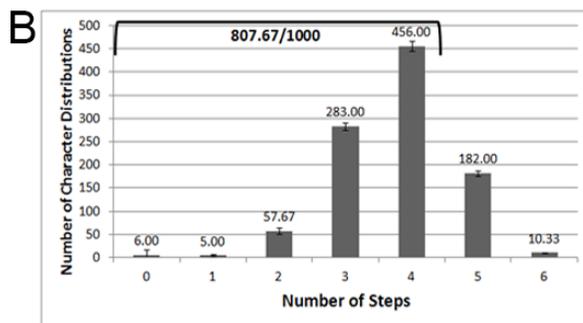
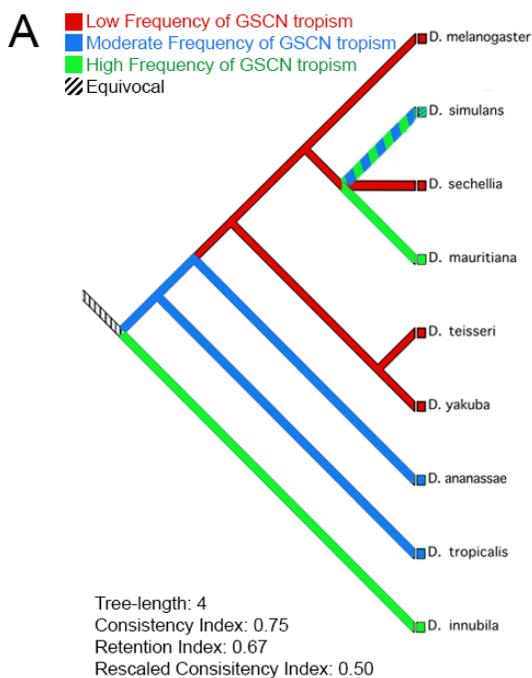


Figure 3.9: *Wolbachia* strain determines differential targeting of the germline stem cell niche

Wolbachia strains *wMau* and *wSh* were introgressed into *D. sechellia* and *D. mauritiana*, respectively. **A.** Representative images of *Wolbachia* niche targeting in the parental (top) and F5 hybrid (bottom) host germaria. The red and green arrows represent the direction of *Wolbachia* transfer. The male genital arch is shown to confirm successful introgression of the male genetic background. Scale Bar 10 μm . **B.** Quantification of GSCN targeting in parental (solid bars) and hybrid (striped bars) species (Log reg, $P_{\text{wob}}=4.7 \times 10^{-22}$ and $P_{\text{host}}=0.18$). N: *D. sech wSh*=120, N: *D. mau wSh*=140, N: *D. mau wMau*=100 N: *D. sech wMau*=109. **C.** Voxel density analysis shows that regardless of host genetic background, *Wolbachia wMau* consistently densely infects the GSCN, as compared to *Wolbachia wSh*. Measurements were acquired using MatLab software (N=10 for each). For each species the values were normalized to region 2b. Error bars represent SEM.

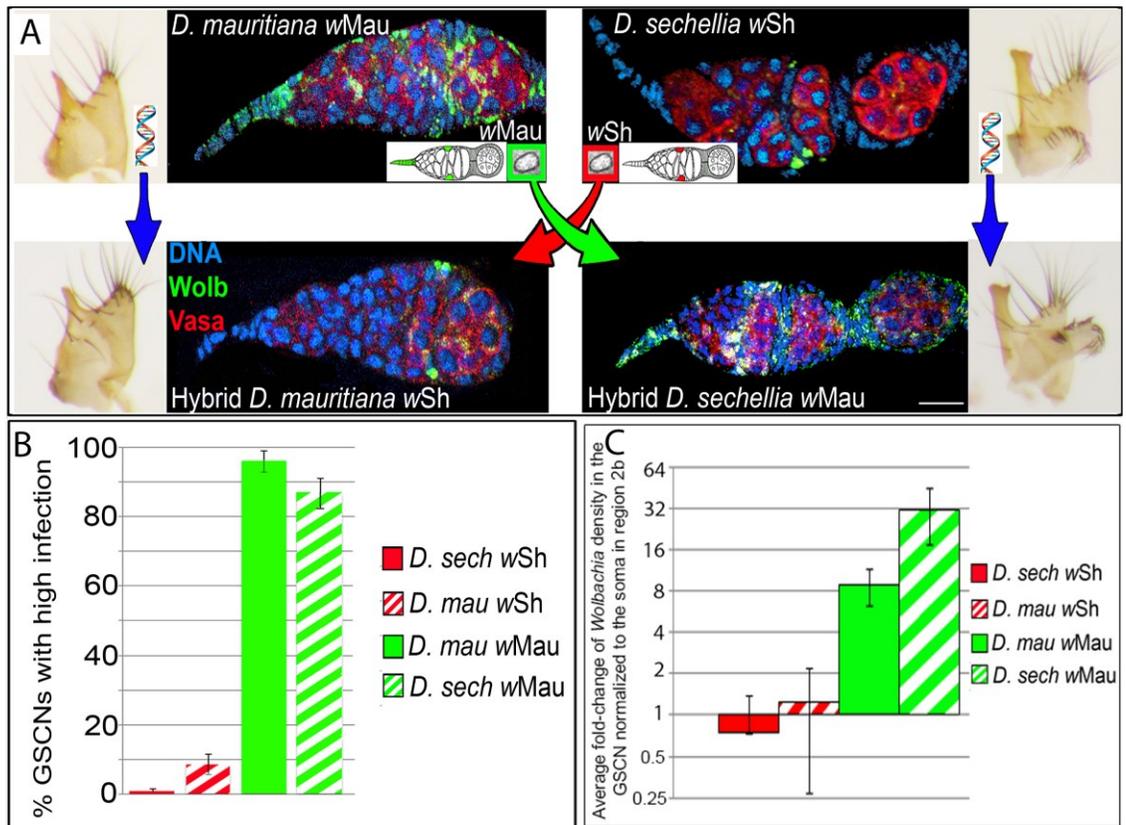


Figure 3.10: Maternally inherited components do not influence GSCN tropism

A. Niche tropism of *wMel* transinfected into *D. simulans* via embryonic microinjection confirms results from hybrid introgression crosses. Scale Bar 10 μm . **B.** *D. simulans* naturally infected with *wRi* targets the GSCN at a higher frequency (N=99) than either *D. simulans* transinfected with *wMel* (N=142) or *D. melanogaster* naturally infected with *wMel* (N=104). *Wolbachia* strain significantly affects GSCN targeting (or lack of) as compared to host genetic background (Logistic regression, $p=6.7 \times 10^{-7}$ and $p=0.76$, respectively). **C.** Voxel density analysis shows that regardless of host genetic background, *Wolbachia wMel* does not densely infect the GSCN, as compared to *Wolbachia wRi*. Measurements were acquired using MatLab software (N=10 for each). For each species the values were normalized to region 2b. Error bars represent SEM.

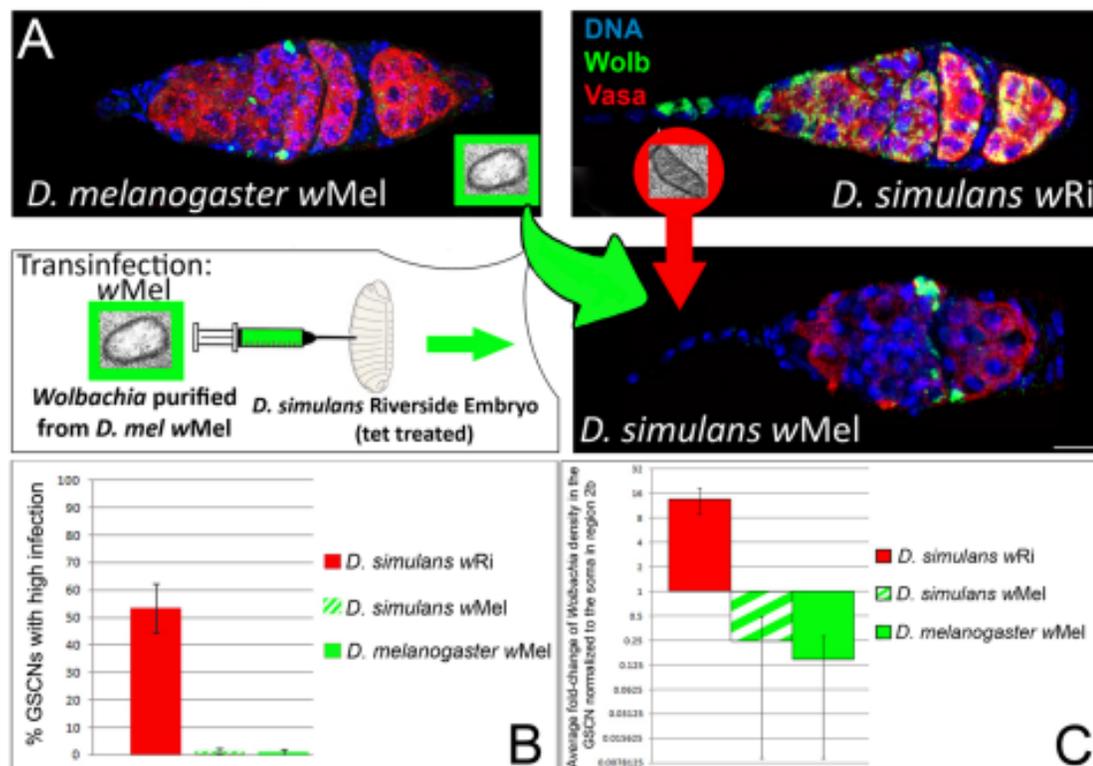


Figure 3.11 *Wolbachia* strain directs patterning within the GSCN

A. *Wolbachia* distribution in GSCN of *w*Ri and *w*No infected *D. simulans* 198,169 (top) and F₅ backcrossed strains (bottom). Scale Bar 10 μ m. **B.** Quantification of parental F₀ (solid bars) and F₅ (striped bars) strains. (Log reg, $P_{wolv}=6.5 \times 10^{-11}$ and $P_{host}=0.54$). $N_{D.sim198 wNo}=120$, $N_{D.sim169 wNo}=122$, $N_{D.sim169 wRi}=100$, $N_{D.sim198 wRi}=130$. Error bars represent SEM.

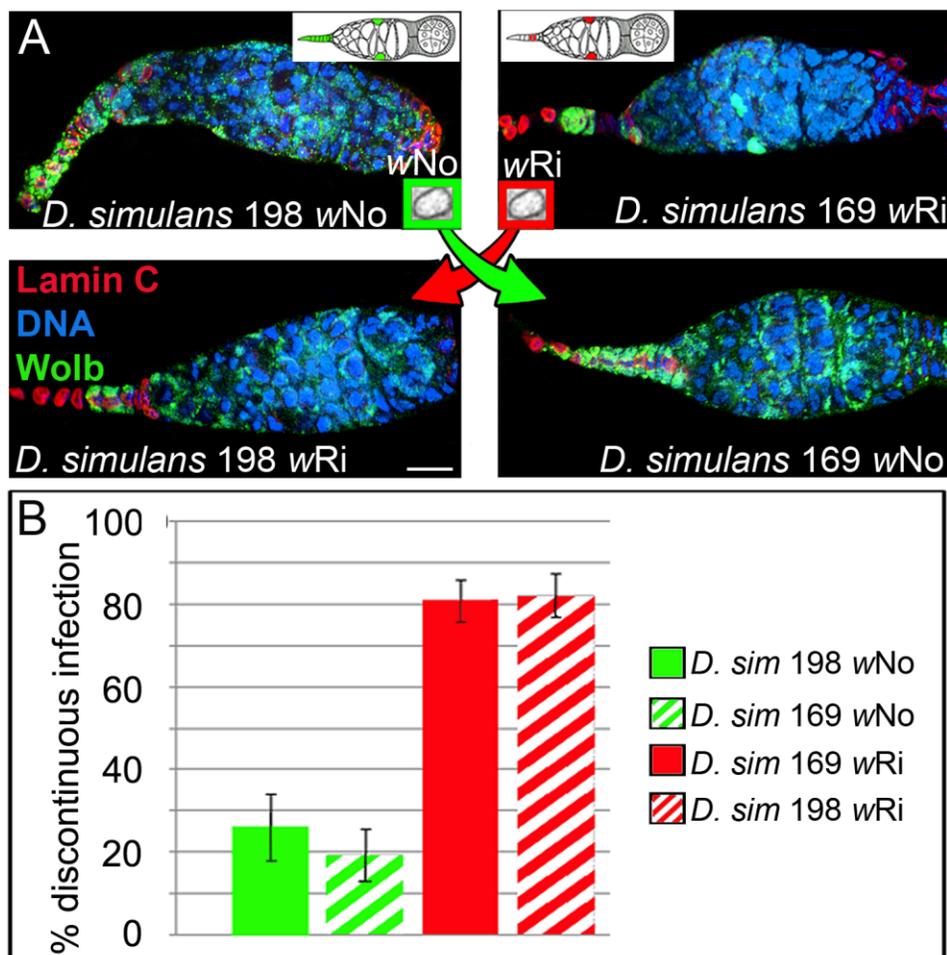


Figure 3.12: Distribution of *Wolbachia* in the germline per region of the germaria

Density of *Wolbachia* infection in the germline per germarial region. Representative images for each *Drosophila-Wolbachia* were analyzed using MatLab to measure the *Wolbachia* pixel density in each of the regions of the germaria as defined in the materials and methods (N=10 for each species). Error bars represent SEM.

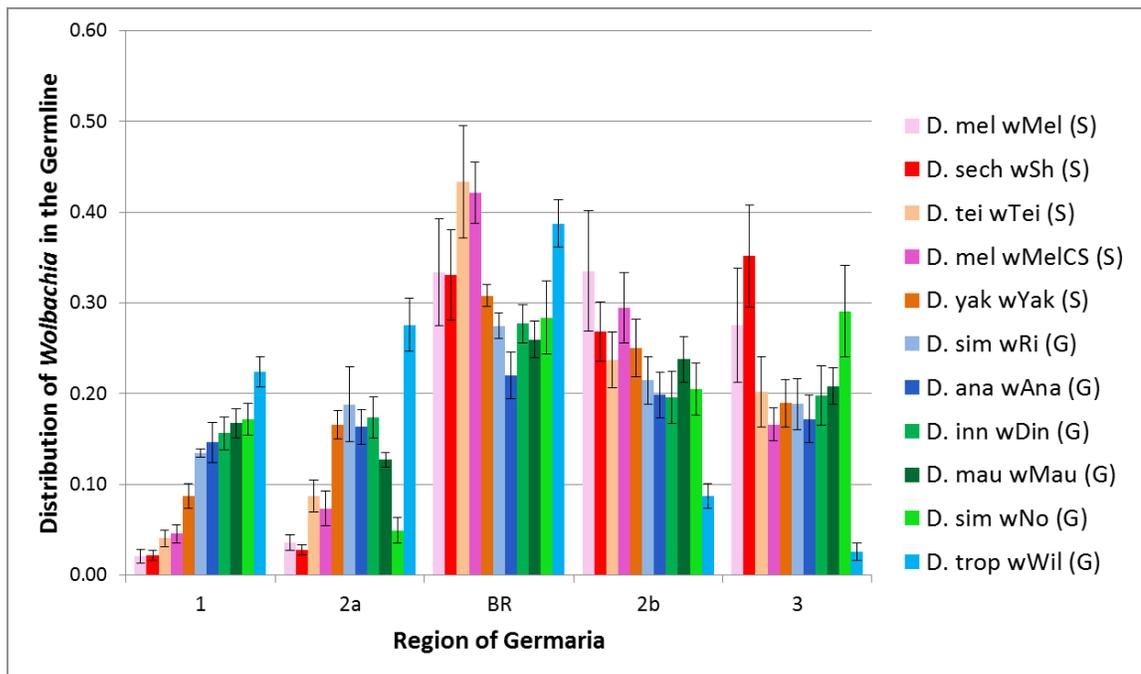


Figure 3.13: *Wolbachia* gradient in the germarium of the various *Drosophila* species

A. Schematic of *Wolbachia* in the germarium. Green and white dots represent *Wolbachia* derived from the GSCN and SSCN, marked in green and white, respectively. Red dots represent *Wolbachia* naturally in the germline. **B-L.** In species with only SSCN tropism (no *Wolbachia* in the GSCN) there is a statistically significant increase of *Wolbachia* density from Region 2a to 2b (as well as in a few species with GSCN tropism; indicated by gradient arrow); quantified in **M** (* $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$; T-test between region 2a and 2b for each sample). **G-L.** As compared to species with only SSCN tropism, there is a statistically higher fraction of *Wolbachia* in Region 1 in species with GSCN tropism (indicated by green asterisk); quantified in **N** ($P = 0.0043$, T-test between *D. simulans* wRi and *D. yakuba* wYak). $N = 10$ germaria each.

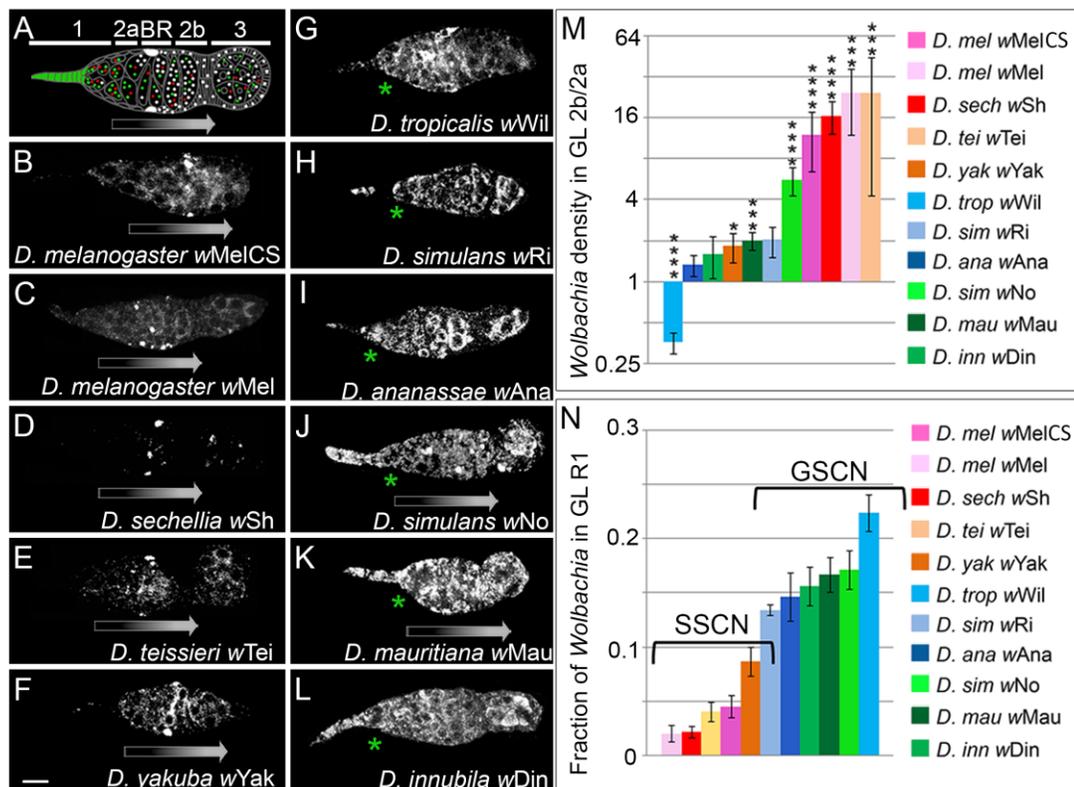


Figure 3.14: *Wolbachia* division in the germaria

A. Representative image showing *Wolbachia wMel* with an abundance of FtsZ puncta in the SSCN of a *D. melanogaster* germarium similar to what is seen at the septum, suggesting that *Wolbachia* in the niche are dividing. Scale bar 10 μ m **B.** Representative confocal image with *Wolbachia* in red, FtsZ in green, and DNA in blue. *Wolbachia* is dividing if FtsZ is clearly localized to the center of the *Wolbachia* cell (red arrowhead, magnification **B'**). Non-dividing *Wolbachia* do not have FtsZ localized to the center (blue arrowhead). *Wolbachia* in clumps (yellow arrowhead) were not counted because it was not possible to determine the FtsZ localization. **C.** Quantification of the fraction of *Wolbachia wSh* dividing in regions 2a and 2b of *D. sechellia* germaria (N=35 germaria from 7 ovaries). A total of 981 individual *Wolbachia* cells were counted, and the fraction of those *Wolbachia* that were dividing was calculated. There is no statistically significant difference in the fraction of *Wolbachia* dividing between regions 2a and 2b (P= 0.41, two-tailed t-test).

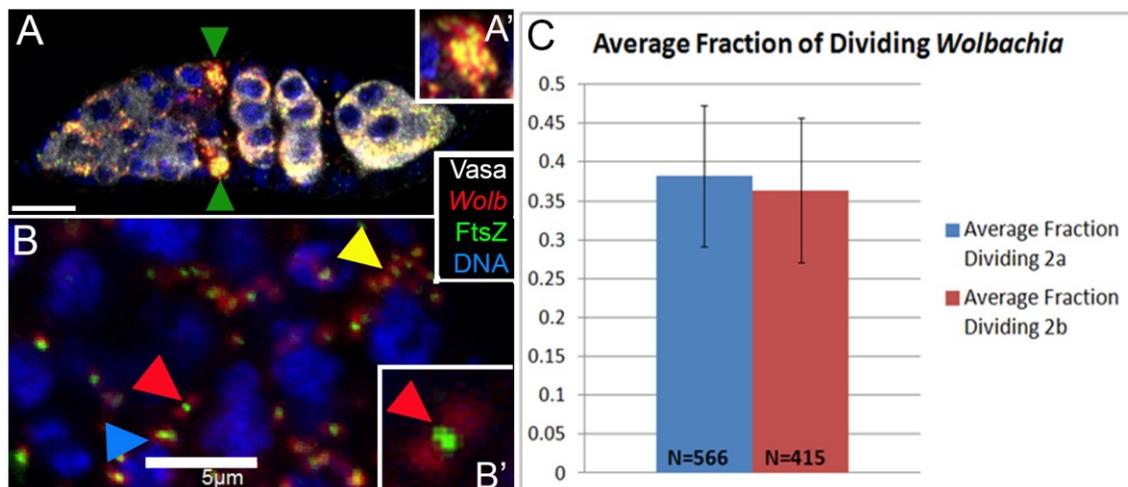


Figure 3.15: Model for *Wolbachia* transmission from the stem cell niches into the germline

Wolbachia originating from the SSCN, rather than from the GSCN, is more likely to invade the germline. **A.** Diagram of egg formation with developmental stages and timeline in days (modified from Drummond-Barbosa *et al.*, 2001; Frydman *et al.*, 2006; He *et al.*, 2011). Developmental timeline is colored according to potential for *Wolbachia* transmission from the GSCN and escort cells (blue, days 0 to 2.5) or from the SSCN, either directly (red, day 2.5) or indirectly (yellow, days 2.5 to 7.3). **B.** Diagram of potential sources of *Wolbachia* transmission into the germ cells from somatic cells present in the germarium and representative egg chambers. **C.** Magnification of *Wolbachia* transfer from the GSCN (dark blue arrows) or the escort cells (light blue arrows). **D.** Magnification of *Wolbachia* transmission directly from the SSCN (red arrows). **E-G.** The somatic tissue infected with *Wolbachia* originating from the SSC can indirectly transmit *Wolbachia* into the germline for the rest of egg development (yellow arrows).

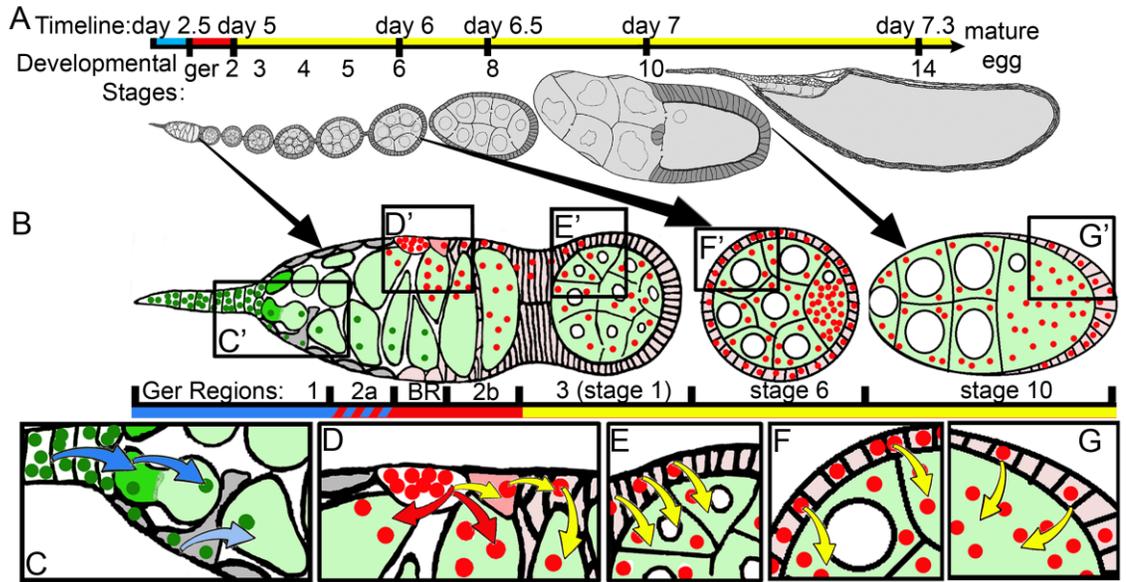


Figure 3.16: Potential passage of *Wolbachia* from the follicle cells into the germline

A. Electron micrograph showing an early stage 8 egg chamber. *Wolbachia* (orange arrowhead) are present at high concentrations in the oocyte cytoplasm (O_{cyt}). *Wolbachia* also infect follicle cells (blue arrowheads). During vitellogenesis, there is endocytosis of yolk proteins and lipid droplets (yellow arrowhead) by the oocyte. A significant fraction of yolk proteins and lipid droplets enter the oocyte from the surrounding follicle cells (FC), suggesting that *Wolbachia* present in the FC may also be actively taken up by the oocyte (red arrowhead). A'. Magnification of region outlined in red showing the *Wolbachia* found entering the oocyte from the apical side of the FC. Mitochondria are indicated for comparison (green arrows). NC, nurse cells; O_{cyt} , oocyte cytoplasm; O_{nuc} , oocyte nucleus; FC, follicle cells.

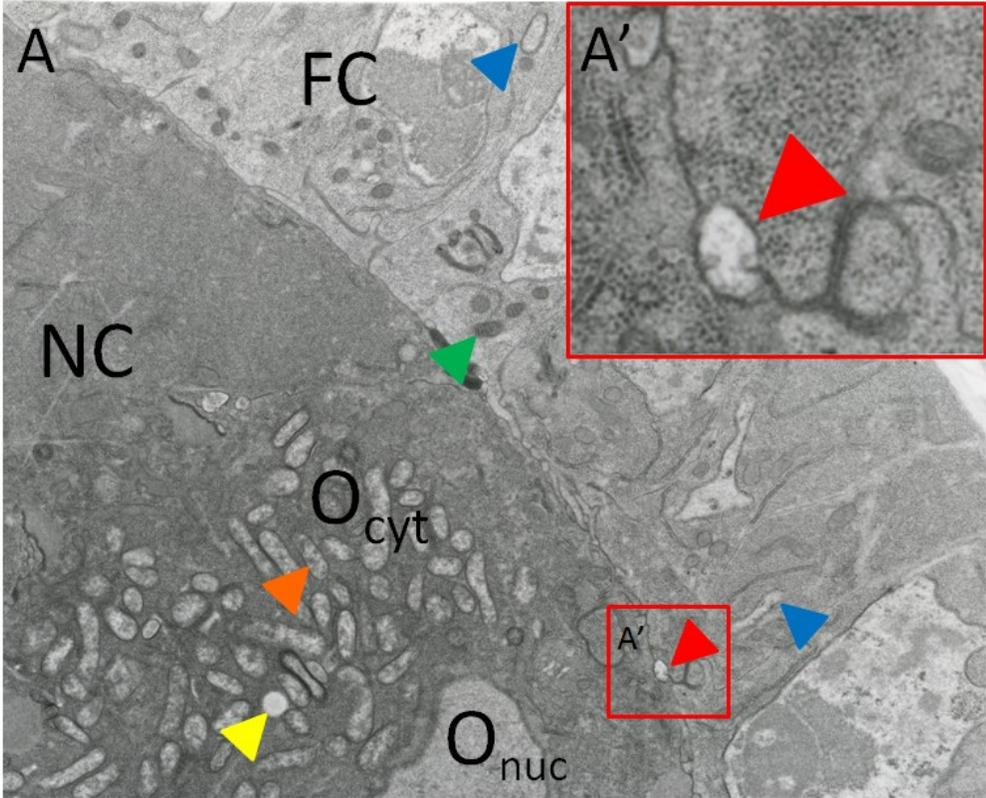


Table 3.1: Frequency of *Wolbachia* stem cell niche tropism in diverse *Drosophila*-*Wolbachia* pairs

<i>Drosophila</i> Species	<i>Wolbachia</i> Strain	# Ovaries	Total # Germaria	% High GSCN \pm SEM	% High SSCN \pm SEM
<i>D. sechellia</i>	wSh	12	120	0.83 \pm 0.83	89.17 \pm 2.88
<i>D. melanogaster</i>	wMel	10	104	0.96 \pm 1.00	93.27 \pm 2.60
<i>D. melanogaster</i>	wMelCS	11	110	0.91 \pm 0.95	94.55 \pm 2.07
<i>D. yakuba</i>	wYak	10	103	0.97 \pm 0.91	82.52 \pm 4.91
<i>D. teissieri</i>	wTei	11	110	3.64 \pm 2.03	84.55 \pm 4.12
<i>D. tropicalis</i>	wWil	11	110	32.73 \pm 7.02	97.27 \pm 1.41
<i>D. ananassae</i>	wAna	9	92	51.09 \pm 5.27	94.57 \pm 1.64
<i>D. simulans</i>	wRi	10	99	53.54 \pm 8.78	83.84 \pm 4.99
<i>D. mauritiana</i>	wMau	10	100	96.00 \pm 3.22	99.00 \pm 1.05
<i>D. innubila</i>	wDin	11	108	96.30 \pm 1.56	99.07 \pm 0.77
<i>D. simulans</i>	wNo	14	138	99.28 \pm 0.71	94.93 \pm 2.55

Tropism for the GSCN and BR of seven day old flies was assessed via visual quantification of confocal images. Approximately 10 germaria from each ovary (and one ovary from each fly) were analyzed and an average frequency of niches highly infected was calculated.

Table 3.2: Average density of *Wolbachia* in the soma of each region of the germarium for each *Drosophila-Wolbachia* pair analyzed

	GSCN	1	2a	BR	2b	3
<i>D. mel</i> wMel	0.18 ± 0.14	0.05 ± 0.03	2.98 ± 1.63	34.59 ± 7.29	10.36 ± 4.93	2.11 ± 1.19
<i>D. mau</i> wMau	103.84 ± 23.23	46.50 ± 13.06	32.44 ± 11.35	54.45 ± 16.27	20.22 ± 4.50	18.22 ± 4.75
<i>D. inn</i> wDin	331.58 ± 85.34	67.86 ± 19.31	52.13 ± 18.63	142.83 ± 39.43	61.86 ± 18.46	51.04 ± 19.79
<i>D. sech</i> wSh	0.25 ± 0.21	0.53 ± 0.20	0.26 ± 0.15	6.06 ± 1.66	0.60 ± 0.27	0.47 ± 0.22
<i>D. sim</i> wRi	34.50 ± 11.64	4.90 ± 1.22	2.02 ± 0.60	11.03 ± 4.92	1.77 ± 1.05	0.27 ± 0.15
<i>D. sim</i> wNo	105.74 ± 22.64	19.69 ± 5.81	5.37 ± 2.22	29.83 ± 9.49	9.07 ± 2.80	17.70 ± 7.02
<i>D. tei</i> wTei	0.15 ± 0.09	0.47 ± 0.19	0.95 ± 0.39	12.75 ± 9.49	1.01 ± 0.34	1.22 ± 0.52
<i>D. trop</i> wWil	4.72 ± 1.63	3.08 ± 0.99	1.98 ± 0.81	9.57 ± 2.77	0.98 ± 0.61	0.07 ± 0.05
<i>D. yak</i> wYak	0.84 ± 0.24	5.12 ± 1.73	5.70 ± 1.37	26.11 ± 6.18	4.78 ± 2.19	1.59 ± 0.48
<i>D. mel</i> wMelCS	1.87 ± 0.92	3.18 ± 1.33	7.84 ± 3.45	85.30 ± 18.76	12.96 ± 4.58	2.69 ± 0.97
<i>D. ana</i> wAna	114.12 ± 50.08	114.49 ± 76.14	124.69 ± 63.04	145.52 ± 72.42	79.60 ± 36.55	57.12 ± 27.37

MatLab image analysis was conducted on representative images from each *Drosophila-Wolbachia* pair to quantify *Wolbachia* pixel density in the soma of each region of the germarium. Although part of region 1, the GSCN was considered its own region for the purposes of this analysis. Values are represented ± SEM.

Table 3.3: Average fraction of *Wolbachia* density in the soma per region of the germarium of representative images

	1	2a	BR	2b	3
<i>D. mel</i> wMel	0.00 ± 0.00	0.06 ± 0.03	0.74 ± 0.03	0.16 ± 0.04	0.03 ± 0.01
<i>D. mau</i> wMau	0.29 ± 0.04	0.16 ± 0.02	0.28 ± 0.03	0.14 ± 0.02	0.13 ± 0.02
<i>D. inn</i> wDin	0.23 ± 0.05	0.11 ± 0.03	0.38 ± 0.03	0.16 ± 0.02	0.11 ± 0.02
<i>D. sech</i> wSh	0.07 ± 0.02	0.02 ± 0.01	0.79 ± 0.04	0.07 ± 0.02	0.05 ± 0.02
<i>D. sim</i> wRi	0.37 ± 0.08	0.13 ± 0.03	0.46 ± 0.06	0.04 ± 0.02	0.01 ± 0.00
<i>D. sim</i> wNo	0.26 ± 0.04	0.06 ± 0.02	0.42 ± 0.07	0.10 ± 0.02	0.17 ± 0.04
<i>D. tei</i> wTei	0.04 ± 0.01	0.08 ± 0.02	0.65 ± 0.08	0.13 ± 0.03	0.11 ± 0.04
<i>D. trop</i> wWil	0.27 ± 0.05	0.12 ± 0.02	0.58 ± 0.06	0.03 ± 0.01	0.00 ± 0.00
<i>D. yak</i> wYak	0.10 ± 0.02	0.18 ± 0.03	0.59 ± 0.06	0.08 ± 0.03	0.05 ± 0.01
<i>D. mel</i> wMelCS	0.03 ± 0.01	0.07 ± 0.03	0.76 ± 0.05	0.11 ± 0.03	0.03 ± 0.01
<i>D. ana</i> wAna	0.18 ± 0.02	0.24 ± 0.03	0.28 ± 0.02	0.18 ± 0.02	0.12 ± 0.01

Average fraction per region of total *Wolbachia* in the germaria was calculated from Table 3.2 to normalize the data. Values are represented ± SEM.

Table 3.4: Quantification of stem cell niche tropism

	SSCN tropism (BR/2b \pm SEM)	GSCN tropism (GSCN/2b \pm SEM)
<i>D. mel</i> wMel	9.71 \pm 3.38	0.15 \pm 0.14
<i>D. mau</i> wMau	3.02 \pm 0.86	8.94 \pm 2.63
<i>D. inn</i> wDin	2.92 \pm 0.64	11.66 \pm 4.77
<i>D. sech</i> wSh	58.58 \pm 39.24	0.74 \pm 0.65
<i>D. sim</i> wRi	4.40 \pm 1.26	13.64 \pm 4.79
<i>D. sim</i> wNo	5.23 \pm 2.66	23.92 \pm 9.12
<i>D. tei</i> wTei	10.40 \pm 4.45	0.14 \pm 0.07
<i>D. trop</i> wWil	27.43 \pm 11.33	26.45 \pm 16.05
<i>D. yak</i> wYak	21.11 \pm 8.79	0.26 \pm 0.11
<i>D. mel</i> wMelCS	54.61 \pm 33.08	0.19 \pm 0.11
<i>D. ana</i> wAna	1.95 \pm 0.36	2.5 \pm 0.97

From MatLab image analysis, enrichment of *Wolbachia* in the stem cell niches was determined by normalizing *Wolbachia* density in the niche region to region 2b of the soma and averaged across samples for each species as a baseline of *Wolbachia* levels in the soma.

For GSCN tropism, data for the species that were considered targeting are in bold.

Table 3.5: Average density of *Wolbachia* in the germline of each region of the germarium for each *Drosophila-Wolbachia* pair analyzed

	1	2a	BR	2b	3
<i>D. mel</i> wMel	3.45 ± 2.21	4.70 ± 3.05	22.18 ± 9.60	52.37 ± 39.21	35.70 ± 25.81
<i>D. mau</i> wMau	37.57 ± 8.38	27.62 ± 5.59	59.20 ± 13.68	51.91 ± 10.77	50.11 ± 13.37
<i>D. inn</i> wDin	233.52 ± 45.47	254.49 ± 50.84	409.11 ± 70.78	337.24 ± 83.19	356.19 ± 101.73
<i>D. sech</i> wSh	1.04 ± 0.34	1.15 ± 0.29	13.92 ± 3.50	15.55 ± 6.64	14.40 ± 2.79
<i>D. sim</i> wRi	12.63 ± 2.31	17.03 ± 4.08	28.38 ± 7.02	22.02 ± 6.16	19.31 ± 5.41
<i>D. sim</i> wNo	31.99 ± 8.28	9.24 ± 2.52	45.78 ± 10.37	35.57 ± 9.82	68.64 ± 21.82
<i>D. tei</i> wTei	2.51 ± 0.80	5.02 ± 1.43	24.71 ± 11.59	14.39 ± 4.83	15.80 ± 4.98
<i>D. trop</i> wWil	10.51 ± 3.57	13.29 ± 4.17	17.18 ± 4.89	4.96 ± 2.12	2.30 ± 1.60
<i>D. yak</i> wYak	21.30 ± 7.16	42.19 ± 14.58	78.72 ± 31.38	67.83 ± 31.97	46.82 ± 16.85
<i>D. mel</i> wMelCS	13.73 ± 5.43	27.25 ± 12.36	104.04 ± 40.99	112.01 ± 67.78	45.84 ± 22.47
<i>D. ana</i> wAna	281.02 ± 142.94	296.11 ± 122.42	413.16 ± 162.23	357.75 ± 143.33	334.42 ± 133.70

MatLab image analysis was conducted on representative images from each *Drosophila-Wolbachia* pair to quantify *Wolbachia* pixel density in the germline of each region of the germarium. Values are represented ± SEM.

Table 3.6: *Wolbachia* enrichment in the germline after passage through the border region

	<i>Wolbachia</i> Density 2b/2a germline
<i>D. mel wMel</i>	24.50 ± 12.52
<i>D. mau wMau</i>	2.00 ± 0.29
<i>D. inn wDin</i>	1.60 ± 0.54
<i>D. sech wSh</i>	16.65 ± 4.45
<i>D. sim wRi</i>	2.03 ± 0.52
<i>D. sim wNo</i>	5.62 ± 1.36
<i>D. tei wTei</i>	24.65 ± 20.38
<i>D. trop wWil</i>	0.36 ± 0.06
<i>D. yak wYak</i>	1.86 ± 0.44
<i>D. mel wMelCS</i>	12.07 ± 5.65
<i>D. ana wAna</i>	1.33 ± 0.23

Wolbachia density in region 2b was compared to density in region 2a and then averaged across samples to assess *Wolbachia* enrichment in the germline after passage through the border region containing the highly infected SSCN.

Table 3.7: Average fraction of *Wolbachia* density in the germline per region of the germarium of representative images

	1	2a	BR	2b	3
<i>D. mel</i> wMel	0.02 ± 0.01	0.04 ± 0.01	0.33 ± 0.06	0.34 ± 0.07	0.28 ± 0.06
<i>D. mau</i> wMau	0.17 ± 0.02	0.13 ± 0.01	0.26 ± 0.02	0.24 ± 0.03	0.21 ± 0.02
<i>D. inn</i> wDin	0.16 ± 0.02	0.17 ± 0.02	0.28 ± 0.02	0.20 ± 0.03	0.20 ± 0.03
<i>D. sech</i> wSh	0.02 ± 0.01	0.03 ± 0.01	0.33 ± 0.05	0.27 ± 0.03	0.35 ± 0.06
<i>D. sim</i> wRi	0.13 ± 0.00	0.19 ± 0.04	0.27 ± 0.01	0.21 ± 0.03	0.19 ± 0.03
<i>D. sim</i> wNo	0.17 ± 0.02	0.05 ± 0.01	0.28 ± 0.04	0.20 ± 0.03	0.29 ± 0.05
<i>D. tei</i> wTei	0.04 ± 0.01	0.09 ± 0.02	0.43 ± 0.06	0.24 ± 0.03	0.20 ± 0.04
<i>D. trop</i> wWil	0.22 ± 0.02	0.28 ± 0.03	0.39 ± 0.03	0.09 ± 0.01	0.03 ± 0.01
<i>D. yak</i> wYak	0.09 ± 0.01	0.17 ± 0.02	0.31 ± 0.01	0.25 ± 0.03	0.19 ± 0.03
<i>D. mel</i> wMelCS	0.05 ± 0.01	0.07 ± 0.02	0.42 ± 0.03	0.29 ± 0.04	0.17 ± 0.02
<i>D. ana</i> wAna	0.15 ± 0.02	0.16 ± 0.02	0.22 ± 0.03	0.20 ± 0.03	0.17 ± 0.03

The average fraction of *Wolbachia* density per region was calculated from Table 3.5 to normalize the data. Values are represented ± SEM.

Table 3.8: Quantification of *Wolbachia* stem cell niche tropism in *Drosophila mauritiana* and *Drosophila sechellia* hybrids

<i>Drosophila</i> Species	<i>Wolbachia</i> Strain	# Ovaries	Total # Germaria	%High GSCN \pm SEM
<i>D. sechellia</i>	wSh	12	120	0.83 \pm 0.83
<i>D. mauritiana</i>	wSh	14	140	8.57 \pm 2.94
<i>D. mauritiana</i>	wMau	10	100	96 \pm 3.05
<i>D. sechellia</i>	wMau	10	109	86.87 \pm 4.35

Tropism for the GSCN of seven day old flies was assessed via visual quantification of confocal images. Approximately 10 germaria from each ovary were analyzed and an average frequency of niches highly infected was calculated. Bolded lines are *Wolbachia* strains backcrossed into a non-native host genetic background.

Table 3.9: Quantification of *Wolbachia* stem cell niche tropism in *Drosophila simulans* and *Drosophila melanogaster* hybrids

<i>Drosophila</i> Species	<i>Wolbachia</i> Strain	# Ovaries	Total # Germaria	%High GSCN \pm SEM
<i>D. simulans</i>	wRi	10	99	53.54 \pm 8.63
<i>D. simulans</i>	wMel	14	142	1.41 \pm 0.93
<i>D. melanogaster</i>	wMel	10	104	0.96 \pm 1.00

Tropism for the GSCN of seven day old flies was assessed via visual quantification of confocal images. Approximately 10 germaria from each ovary were analyzed and an average frequency of niches highly infected was calculated. Bolded lines are *Wolbachia* strains transinfected into a non-native host genetic background.

Table 3.10: Quantification of *Wolbachia* stem cell niche tropism in *Drosophila simulans* hybrids

<i>Drosophila</i> Species	<i>Wolbachia</i> Strain	# Germaria	%High GSCN ± SEM	% Discontinuous ± SEM
<i>D. simulans</i> 198	wNo	120	90.00 ± 2.75	25.93 ± 7.73
<i>D. simulans</i> 169	wNo	122	93.44 ± 2.22	19.30 ± 6.31
<i>D. simulans</i> 169	wRi	100	75.00 ± 6.49	81.00 ± 5.06
<i>D. simulans</i> 198	wRi	130	60.77 ± 5.00	82.28 ± 5.27

Tropism for the GSCN of seven day old flies was assessed via visual quantification of confocal images. Approximately 10 germaria from each ovary were analyzed and an average frequency of niches highly infected was calculated. Bolded lines are *Wolbachia* strains backcrossed into a non-native host genetic background.

CHAPTER 4

Wolbachia Stem Cell Niche Tropism in the Testis

Portions of this chapter were previously published in (Toomey *et al.*, 2014)

4.1 Introduction

The evolutionary interests of males and females are frequently divergent. Sexual conflict arises when phenotypes that enhance the reproductive success of one sex reduces the fitness of the other sex (Chapman *et al.*, 2003). A well-characterized example in *Drosophila* is sperm competition between males. Sperm competition results in rapid evolution of sperm proteins which up-regulate females' egg-laying rate and reduces their desire to re-mate with another male. However, these proteins also shorten the female's lifespan, reducing her fitness (reviewed by Avila *et al.*, 2011).

Vertically transmitted reproductive parasites, such as *Wolbachia*, can also cause sexually divergent phenotypes in males and females. *Wolbachia* are obligate intracellular bacteria present in a large fraction of insects, as well as spiders, mites, crustaceans, and filarial worms. They are primarily vertically transmitted from mother to offspring in a manner analogous to mitochondrial inheritance, although there is extensive evidence of horizontal transmission in nature (Schilthuizen *et al.*, 1997; Baldo *et al.*, 2008). For intracellular bacteria, vertical transmission often favors infected females, which is also the case for *Wolbachia* (Werren, 2011). There are several *Wolbachia*-induced phenotypes favoring the infected female, including parthenogenesis, feminization, male killing, and cytoplasmic incompatibility (Werren *et al.*, 2008). Each of these phenotypes ultimately

results in the spread of more infected female hosts. In such cases, maternally transmitted bacteria can act as selfish genetic elements driving sexual conflict (Werren, 2011).

For successful vertical transmission, *Wolbachia* need to be present in the eggs laid by infected females. It has been shown in *Drosophila* that *Wolbachia* display a strong tropism for the germline, in particular, the oocyte, to ensure a high percentage of vertical transmission (Hadfield *et al.*, 1999; Veneti *et al.*, 2004; Ferree *et al.*, 2005; Serbus *et al.*, 2007). Although vertical transmission is prevalent, *Wolbachia* also can spread horizontally across individuals and species (Boyle *et al.*, 1993; Vavre *et al.*, 1999; Baldo *et al.*, 2008). Colonization of the germline is a prerequisite for the infection to become successfully established in a population. We have previously shown that upon recent infection, *Wolbachia* colonize the stem cell niches in the *Drosophila* ovary, favoring vertical transmission after horizontal transfer (Frydman *et al.*, 2006). Furthermore, stem cell niche tropism in the ovary is a highly evolutionarily conserved phenotype across the *Drosophila* genus, present in 100% of ovaries analyzed (Toomey *et al.*, 2013). *Wolbachia* also infect the putative stem cell niches in the ovaries of other species, such as the bedbug and leafhopper (Hosokawa *et al.*, 2010; Sacchi *et al.*, 2010), indicating that the selective pressure for *Wolbachia* targeting of ovarian stem cell niches to favor transmission extends beyond the *Drosophila* genus.

Wolbachia have also been shown to display tropism to the stem cell niche present in the testis in *D. mau* (Fast *et al.*, 2011). However, the conservation of this phenotype across the *Drosophila* genus is unknown. Here we show that the evolutionary conservation of stem cell niche tropism present in females is not maintained in the male lineage. In fact,

Wolbachia niche tropism in the testis, compared to the female results, represents a pronounced sexual dimorphism in the evolutionary history of *Wolbachia* stem cell niche tropism. Furthermore, we have determined that both *Wolbachia* and host factors modulate hub tropism in this system. Finally, we show that closely related *Wolbachia* strains infecting the same host differ significantly in the densities at which they colonize the hub, indicating that hub tropism is a rapidly diverging phenotype in males.

4.2 *Wolbachia* targeting of the hub in the *Drosophila* testis is not pervasive

In the testis, the germline stem cells (GSCs) and cyst stem cells (CySCs) reside at the “hub”, a structure at the apical tip of the testis (Figure 4.1A). The hub is a group of 10 to 16 somatically derived cells forming the microenvironment supporting the stem cells, referred to as the niche (Hardy *et al.*, 1979). It has been shown that the GSCs receive maintenance signals from both the hub and the CySCs, hence both are considered to be part of the stem cell niche for the GSCs. However for the context of this study, niche tropism in the testis refers to *Wolbachia* infection of the hub only. To investigate whether *Wolbachia* niche tropism is as pervasive in the hub, as previously shown in the ovary (Toomey *et al.*, 2013), we surveyed various *Drosophila* species infected with different strains of *Wolbachia* (Figure 4.1 B-L, Table 4.1).

Using confocal imaging and immunohistochemistry, we analyzed the density of *Wolbachia* infection in the hub cells as compared to the density of *Wolbachia* in the surrounding tissue (see Material and Methods). We found that *Wolbachia* target the hub at varying frequencies and densities across the *Drosophila* genus, separating into three statistically distinct groups (Figure 4.1, Table 4.1). Three out of nine species showed little

to no (0-2.33%) *Wolbachia* infection in the hub (Figure 4.1 H-J, quantification in K), indicating that hub tropism is not pervasive across the *Drosophila* genus. Six out of nine species analyzed, however, did have *Wolbachia* tropism to the hub, ranging from 17% of niches infected to 95% of niches infected (Figure 4.1 B-G, K). The six *Drosophila* species-*Wolbachia* strain pairs with hub tropism fall into two groups with significantly different frequencies and densities of tropism. Three had high frequencies (65-83%) and densities (4.3-6.3-fold higher than surrounding germline and soma) of hub infection: *D. ananassae* wAna, *D. melanogaster* wMel, and *D. mauritiana* wMau. Three had moderate frequencies (17-30%) of *Wolbachia* tropism to the hub: *D. yakuba*, wYak, *D. tropicalis* wWil, and *D. simulans* wRi. In the ovary, tropism to the somatic stem cell niche is found at high frequencies in every individual of all *Drosophila* species analyzed (Toomey *et al.*, 2013). In contrast, tropism for the hub is found in only a fraction of the species analyzed.

4.3 Hub targeting does not correlate with germline stem cell niche tropism pattern in the ovary

Similar to the results for hub tropism, the frequency of tropism to the germline stem cell niche (GSCN) in the ovary was shown to be variable across the *Drosophila* genus (Figure 4.2A and (Toomey *et al.*, 2013)). We reasoned that *Wolbachia* tropism to the hub in the testis could simply be a byproduct of GSCN targeting in the ovary. However, the presence of hub tropism does not correlate with the presence GSCN tropism (Table 4.2, Correlation Test, $p=0.773$). Although tropism in males and females correlates in some strains (5 out of 9, i.e., wMau displays high frequencies of both hub tropism and GSCN tropism and wSh does not have tropism to either the hub or the GSCN), there are others

where there is no correlation (4 out of 9). The *Wolbachia* strain displaying one of the highest frequencies of GSCN tropism in the ovary (*w*No, 99% (Toomey *et al.*, 2013)), displays no tropism to the hub (0%, Figure 4.1 I and K). Conversely, a *Wolbachia* strain displaying a high frequency of tropism to the hub (*w*Mel, 71%, Figure 4.1 C and K) does not target the GSCN in the ovary (1%, (Toomey *et al.*, 2013)). These data reveal that *Wolbachia* stem cell niche tropism in the male does not correlate with GSCN tropism in the female.

4.4 Hub tropism phenotype is independent of host and bacterial phylogenies

Previously, we have shown that the pattern of GSCN tropism is evolutionarily conserved across the *Wolbachia* lineage ((Toomey *et al.*, 2013) and Figure 4.2). To assess whether hub tropism was also conserved across the *Wolbachia* lineage, we aligned the frequencies of hub tropism on the *Wolbachia* phylogenetic tree (Figure 4.2). We quantified the correlation of hub tropism pattern with the *Wolbachia* phylogeny using a computer simulation model of randomized character distributions to compare with the distribution of niche tropism pattern on each of the phylogenies, as previously described (Toomey *et al.*, 2013). We found that it is highly probable that the distribution of hub tropism is independent of the *Wolbachia* phylogeny (Figure 4.3). Similarly, when we compared hub tropism to the *Drosophila* phylogeny, we found no clear correlation between the two (Figure 4.4). Quantification of the relationship revealed that frequency of hub tropism bears no correlation with the *Drosophila* phylogeny (Figure 4.5).

4.5 Hub tropism does not correlate with cytoplasmic incompatibility

An important *Wolbachia* related phenotype that also bears no correlation with host or microbial phylogenies is cytoplasmic incompatibility (CI). CI is a reproductive phenotype resulting in reduced embryo hatching when a *Wolbachia* infected male mates with an uninfected female. We examined the possibility of a correlation between tropism to the hub and CI by comparing our tropism data to previously published reports on the levels of CI across the *Drosophila* genus (Table 4.3) (Bourtzis *et al.*, 1996; Van Meer *et al.*, 1999; Charlat *et al.*, 2002; Veneti *et al.*, 2003; Zabalou *et al.*, 2004). This analysis shows that some species with high levels of CI have different levels of tropism (i.e., *wSh* and *wRi* have 0% and 17% hub tropism, respectively). Conversely, some species with low levels of CI have a wide range hub tropism phenotypes (i.e., *wTei* and *wMau* have 2.3% and 71% hub tropism frequencies, respectively). Although hub tropism is highly divergent even among closely related strains of *Wolbachia*, similar to CI, there does not appear to be a correlation between these two phenotypes (Table 4.3, Correlation test, $p=0.267$).

4.6 Both host and bacterial factors can influence hub tropism

We next aimed to elucidate if host or bacterial factors influence the highly dynamic nature of the hub tropism phenotype. To investigate this question, *Wolbachia* strains backcrossed into a different host were used to assess *Wolbachia* strain versus host background influence on hub tropism, as previously described (Toomey *et al.*, 2013). *D. mauritiana wMau*, which displays hub tropism (Figure 4.1D and Figure 4.6) and *D. sechellia wSh*, which does not display hub tropism (Figure 4.1J and Figure 4.6) and their hybrid offspring were utilized in this study.

Wolbachia strain *wSh*, infecting its native host, *D. sechellia*, and its non-native host, *D. mauritiana*, displays no hub localization, regardless of host genetic background. These data suggest that *Wolbachia wSh* is incapable of hub tropism in either species. However, it does not rule out the possibility that the hosts share a mechanism for excluding *wSh* from the hub. Therefore, a lack of tropism in both hosts cannot provide insight into whether the host or microbe are providing factors contributing to hub tropism.

The analysis of *wMau* hub tropism allows further probing into this question. *Wolbachia* strain *wMau* infecting its native host, *D. mauritiana*, and its non-native host, *D. sechellia*, displays tropism for the hub, suggesting that the *Wolbachia* strain is driving this phenotype. However, the frequency of targeting in the hybrid host is 3-fold lower than in the native host (Figure 4.6 C, green bars). Statistical analysis of frequency data indicates that both host genetic background and *Wolbachia* strain can significantly affect the frequency of hub tropism (Fisher's exact test, $p=8.309 \times 10^{-5}$ and $p=2.267 \times 10^{-10}$, respectively). These results are in contrast to previous data in the ovary where only the *Wolbachia* strain drives tropism. *Wolbachia wMau* target the GSCN in the ovary of both its native and hybrid host at frequency of greater than 80%, regardless of the host genetic background (Toomey *et al.*, 2013). The *wMau* frequency data in the male support the hypothesis that the *Wolbachia* strain is directing hub tropism. However, because the frequency of targeting is not as robust in the hybrid host compared to its native host, a role for the host is also implicated.

In relation to *Wolbachia* density in the hub, the data indicate that *Wolbachia* encoded factors play a major role in both native and hybrid hosts. The overall density at

which *wMau* infect the hub is conserved (Figure 4.6 B and C, native host solid green bar, hybrid host hatched green bar, Table 4.4). Similarly, *wSh* hub titers, compared to the surrounding tissue, is less than 1 in both native and hybrid hosts (Figure 4.6 B and C, native host solid red bar and hybrid host hatched red bar, Table 4.4). Linear regression analysis of density data indicates that the *Wolbachia* strain, rather than the host genetic background, modulates *Wolbachia* density in the hub ($P=0.045$ and $P=0.56$, respectively). With respect to both frequency and density, the overall data reveal that factors encoded by both the host species and the *Wolbachia* strain influence hub tropism in the *Drosophila* testis.

4.7 *Wolbachia* strain factors are sufficient for differences in hub tropism

To further investigate the role of *Wolbachia* on hub tropism, we next analyzed different *Wolbachia* strains in the same host species. We took advantage of *D. simulans*, which is a host to many different *Wolbachia* strains. We investigated two strains of *D. simulans* flies differentially infected with *wRi* and *wNo* and their backcrossed offspring. Flies were backcrossed to account for any genomic divergence between host strains, as previously described (Toomey *et al.*, 2013). *D. simulans* flies infected with *Wolbachia wRi* display hub tropism in about 33% and 43% of hubs analyzed for the parental and backcrossed hosts, respectively (Figure 4.7, Table 4.5). *D. simulans wNo* displays hub tropism infrequently (2% and 15% of hubs highly infected for the parental and backcrossed hosts, respectively, Figure 4.7, Table 4.5). Although the frequencies of hub tropism for each *Wolbachia* strain increase in the backcrossed hosts, the general trend remains, where *wRi* targets the hub at a higher frequency than *wNo*. To quantify the relative contributions of host and bacterial factors towards hub tropism, logistical regression was performed.

Wolbachia factors have a significant effect on hub tropism as compared to no significance of the host genetic background in the *D. simulans* hybrid flies ($P < 0.0001$ and $p = 0.927$ respectively). These results indicate that when host factors are kept constant, *Wolbachia* strain factors are sufficient to significantly modulate the frequency of hub tropism.

4.8 Hub tropism is a rapidly evolving phenotype

In the previous analyses of hybrid crosses, hub tropism of distantly related *Wolbachia* strains were compared, first with different host species (Figure 4.6), then within the same host species (Figure 4.7). These results indicate that although the fly host can play a role in hub tropism, *Wolbachia* can significantly affect tropism on its own. In both cases, we were comparing *Wolbachia* strains from the A and B supergroups. We next investigated if the observed diversity of niche tropism is still present between more closely related *Wolbachia* strains. To address this question, we analyzed hub tropism of several *Wolbachia* strain variants infecting *D. mel* which diverged from a single ancestor within the last 8,000 years (Richardson *et al.*, 2012; Chrostek *et al.*, 2013).

Hub tropism of *wMel*-like (*wMel*, *wMel2*, and *wMel3*) and *wMelCS*-like (*wMelCS*, *wMelCS2*, and *wMelPop*) *Wolbachia* strains were analyzed. These *Wolbachia* strains were introgressed into the same *D. melanogaster* (*D. mel*) genetic background with the same microbiota (Chrostek *et al.*, 2013). The data reveal that the three *wMel*-like *Wolbachia* strains have significantly different tropism phenotypes from the *wMelCS*-like strains (Figure 4.8, Table 4.6). The *wMel*-like strains target the hub at similar frequencies, between 25% and 50%, and at similar densities, about 1.5-fold higher than the surrounding tissue. The *wMelCS*-like strains target the hub at significantly higher frequencies ($P < 0.05$) and

densities ($P < 0.001$) than the *wMel*-like strains. Within the *wMelCS*-like group, *wMelPop* targets the hub at a significantly higher frequency (100%) than *wMelCS2* (77%; $P = 0.005$), but not *wMelCS* (90%). However, *wMelPop* targets at a significantly higher density than both *wMelCS* and *wMelCS2* ($P < 0.0001$). Interestingly, *wMelPop* densities increase to the point where the hub cells burst open in approximately 20% of hubs (Figure 4.9). The finding that the *wMel*-like and *wMelCS*-like *Wolbachia* variants, all derived from a single ancestor only 8,000 years ago, have significantly different frequencies and densities of targeting indicates that hub tropism is a rapidly diverging phenotype.

4.9 Discussion

A fundamental aspect of *Wolbachia*-host interactions is the type of tissue preferentially infected by the bacteria. We have previously shown that *Wolbachia* tropism to the stem cell niches in the female *Drosophila* ovary is important for vertical transmission, and that this tropism is conserved across the *Drosophila* genus. Furthermore, closely related *Wolbachia* strains tend to display the same patterns of tropism in the ovary, indicating the importance of maintaining this phenotype for vertical transmission (Toomey *et al.*, 2013).

If the major role of niche tropism is related to *Wolbachia* transmission, evolutionary theory predicts that there should be reduced selective pressure to maintain niche tropism in males, since *Wolbachia* is not transmitted through the sperm. Patterns of *Wolbachia* niche tropism in the filarial nematode (*B. malayi*, *D. immitis*, *L. sigmondontis*, *M. unguiculatus*, and *O. dewittei japonica*) support this concept, where *Wolbachia* colonization of the distal tip cell (the nematode equivalent of the stem cell niche) and subsequent germline invasion

occurs only in females (Landmann *et al.*, 2012). In agreement, the results shown here indicate a reduced level of conservation of hub tropism phenotype, contrasting with previous observations in females (Toomey *et al.*, 2013). The stem cell niches in the ovary and testis are well characterized and have several signaling pathways in common (Decotto *et al.*, 2005). The robust sexual dimorphism in the evolutionary conservation of niche tropism, indicates that *Wolbachia* could be recognizing novel sex-specific differences in these cells (Gilboa *et al.*, 2004).

Wolbachia-induced host phenotypes related to stem cell biology and testis physiology have been previously described (Bourtzis *et al.*, 1996; Fast *et al.*, 2011). We investigated whether hub tropism correlates with those known *Wolbachia*-related reproductive phenotypes. Because GSCN tropism in the ovary was shown to not be ubiquitous across the *Drosophila* genus, we reasoned that hub tropism could simply be a byproduct of GSCN tropism in the female. However, the frequencies of GSCN and hub tropism only correlate in 5 out of the 10 species and are not statistically significant.

On the cellular level, another phenotype we have previously shown was a *Wolbachia*-dependent increase in the rate of germline stem cell division (GSCD) in the ovaries of *D. mauritiana*. Although a similar trend exists in the *D. mauritiana* testis, the up-regulation of GSCD was not statistically significant, showing a lack of conservation of a phenotype derived in the females to boost their spread (Fast *et al.*, 2011).

A third important *Wolbachia* mediated phenotype, cytoplasmic incompatibility (CI), is a consequence of *Wolbachia* modification of sperm during spermatogenesis, causing embryonic lethality of uninfected eggs fertilized by sperm from infected males

(reviewed by Werren, 1997). Although the precise mechanism is not well understood, the sperm from infected males is modified (mod^+) and an infected egg with the appropriate rescue factor (resc^+) is required for embryo viability (Tram *et al.*, 2002; Pinto *et al.*, 2013). Several lines of evidence suggests that the modification of the sperm occurs at the chromatin level (Breeuwer *et al.*, 1990; O'Neill *et al.*, 1990; Brennan *et al.*, 2012). Extensive analyses of *Wolbachia* population dynamics and localization during spermatogenesis have demonstrated that CI is a non-cell autonomous effect caused by a diffusible *Wolbachia* factor during spermatogenesis (Riparbelli *et al.*, 2007). Interestingly, local factors secreted by the hub can act on the germline stem cell in a non-cell autonomous manner, and have been shown to cooperate with chromatin remodeling complexes towards control of germline stem cell maintenance and differentiation (Cherry *et al.*, 2010). Therefore, we attempted to correlate our tropism data with published data regarding CI levels of several *Wolbachia* strains across the *Drosophila* genus. However, we found no correlation between *Wolbachia* hub tropism and CI. The lack of correlation between hub tropism and CI suggests that *Wolbachia* presence in the hub is not required for the CI effect, and that the *Wolbachia* factor which modifies the sperm occurs later in spermatogenesis.

Literature shows that both the host species and *Wolbachia* strains have rapidly evolving aspects that could contribute to the dynamic evolutionary changes in *Wolbachia* hub targeting shown here. Regarding the host, several testis specific genes, male seminal fluid proteins, and spermatogenesis genes have been shown to be rapidly evolving (Haerty *et al.*, 2007). Furthermore, proteins related to GSC biology are also undergoing recurrent positive selection (Bauer DuMont *et al.*, 2007). From the perspective of the bacteria,

Wolbachia genomic analyses suggest that these bacteria have one of the most highly recombining intracellular bacterial genomes, with many genomic differences between closely related strains (Baldo *et al.*, 2006; Klasson *et al.*, 2009; Baldo *et al.*, 2010; Siozios *et al.*, 2013).

We investigated the relative contribution of both host and bacterial factors towards hub tropism phenotype. Unlike in the ovary where host derived factors did not play a role (Toomey *et al.*, 2013), in the testis, host factors could not be ruled out. When comparing distantly related *Wolbachia* strains and host species (*D. mauritiana* and *D. sechellia* hybrid lines), the data indicate that both host and *Wolbachia* derived factors contribute to the differences in hub tropism. One possibility is that there is selective pressure on the host driving rapid evolution of the hub intracellular environment to counteract negative effects of *Wolbachia* colonization of the testis niche. Although there is no evidence in the literature for positive selection of hub proteins, genes in the neighboring germline stem cell have been shown to be undergoing positive selection (Bauer DuMont *et al.*, 2007; Choi *et al.*, 2014). Independent of differential host factors, we were able to confirm *Wolbachia*'s role in hub tropism. By comparing distantly related *Wolbachia* strains in the same host species (*D. simulans* lines), we were able to confirm that *Wolbachia* derived factors significantly modulate hub tropism.

To assess how quickly this modulation of hub tropism can evolve, we investigated whether closely related *Wolbachia* strains that have recently diverged display diverse hub tropism phenotypes. Several variants of the wMel strain of *Wolbachia* naturally infecting *D. mel* exist (Riegler *et al.*, 2005; Ilinsky, 2013). Due to strict maternal transmission,

congruent *Wolbachia* and mitochondrial lineages made it possible to trace these lineages back to a single common *D. mel* ancestor existing around 8,000 years ago (Richardson *et al.*, 2012; Chrostek *et al.*, 2013). We investigated hub tropism of *wMel*-like (*wMel*, *wMel2*, and *wMel3*) and *wMelCS*-like (*wMelCS*, *wMelCS2*, *wMelPop*) *Wolbachia* strains which have been shown to induce differential protection against viruses (Chrostek *et al.*, 2013). The *wMel*-like and *wMelCS*-like variants can be separated into three statistically distinct groups based on their density of hub infection (1: *wMel*, *wMel2*, and *wMel3*; 2: *wMelCS* and *wMelCS2*; 3: *wMelPop*) indicating that they have distinct cellular tropisms. These data indicate that hub tropism is a rapidly diverging phenotype.

The fast paced changes in the hub tropism phenotype during the evolution of these different *Wolbachia* strains raises questions of what mechanisms are driving these rapid changes and is adaptive evolution occurring. If *Wolbachia* tropism for the hub is causing an unfavorable phenotype in the host, a molecular arms race will result where both the host and microbe will rapidly evolve (Jiggins *et al.*, 2002; Brownlie *et al.*, 2007). We did not find any correlation of hub tropism with CI, germline stem cell division, or with other obvious testis related phenotypes. It is possible that hub tropism may have a phenotypic effect on the host, but at the moment these are unknown and we have no evidence supporting adaptive evolution in response to a host-microbe arms race driving rapid changes in hub tropism in *wMel* strains.

Another possibility is that genetic drift is driving the extreme divergence in hub tropism that we report here. At every generation, from embryonic development through the mature egg, *Wolbachia* undergoes several bottlenecks: only the *Wolbachia* present in the

germplasm of the embryo will colonize the primordial germ cells (Hadfield *et al.*, 1999; Veneti *et al.*, 2004). Within the germline, only the *Wolbachia* present in the oocyte is transmitted to the progeny (Veneti *et al.*, 2004; Ferree *et al.*, 2005; Serbus *et al.*, 2007). This effectively reduces the genetic effective population sizes and increases the rate of fixation of mutations by drift. There are several studies highlighting the role of genetic drift driving high rates of genome sequence evolution in vertically transmitted endosymbionts (reviewed by Moran *et al.*, 2008). The data presented here suggest that mutations that are neutral regarding niche targeting in the female affect niche tropism in the male. If these mutations do not affect *Wolbachia* overall fitness in the females and do not interfere with transmission, they can be fixed by drift and result in significant niche tropism evolution in males.

At the moment it is difficult to identify the specific molecular underpinnings resulting in the differences in niche tropism phenotypes between these strains. A possible molecular player involved in hub tropism is encoded by the gene region known as ‘octomom’. This region was found to be amplified several times in *wMelPop*, and contains genes predicted to be involved in DNA replication. It has been proposed to be responsible for the *wMelPop* overreplication phenotype (Chrostek *et al.*, 2013), although there are conflicting reports (Woolfit *et al.*, 2013). This could explain the highest titers present in *wMelPop* infected hubs. However, there are other unknown factors contributing to the range of hub tropism phenotypes observed in the other *wMelCS*-like and *wMel*-like strains, since they have only once copy of the octomom region. The *wMel* variants are defined by several polymorphic genetic markers (Riegler *et al.*, 2005; Chrostek *et al.*, 2013; Ilinsky,

2013; Woolfit *et al.*, 2013). There are 108 single nucleotide polymorphisms (SNPs), a tandem duplication, and seven insertion-deletion polymorphisms between the *wMel* and *wMelCS*-like (*wMelPop*) strains (Chrostek *et al.*, 2013). Further characterization of niche tropism of different strains in the same host genetic background, together with additional sequencing of diverse strains, will allow the correlation of *Wolbachia* genomic features with patterns of niche tropism. Future identification of *Wolbachia* proteins modulating the different levels of hub tropism will provide insights into the evolutionary mechanism driving this rapid divergence in males and the robust sexual dimorphism of stem cell niche targeting.

Here we presented tropism differences in *Wolbachia* strains well characterized at the genomic level in a *Drosophila* species with a large repertoire of transgenic and genetic tools. These findings provide the foundation to dissect the molecular mechanisms involved in *Wolbachia* hub tropism. Furthermore, the differences in stem cell niche tropism between males and females may reveal sex specific differences in the biology of stem cell niche being recognized by *Wolbachia*. Identification of the *Wolbachia* factors involved in tissue tropism is fundamental in understanding how bacteria spread and infect their hosts in nature and will provide additional tools towards vector and disease control.

Figure 4.1: Diverse *Wolbachia* strains infect the hub of various *Drosophila* species at different frequencies and densities

A. Diagram of the testis apical tip, with cell nuclei in blue. The germline stem cells (GSCs, grey) and cyst stem cells (CySCs, yellow) reside at the hub (red). **B-J.** Representative images of *Wolbachia* (green) hub tropism in 9 *Drosophila* species (hub marker, red; DNA, blue). **K.** Quantification of the frequency of *Wolbachia* hub tropism in each *Drosophila* species (Error bars represent 95% confidence intervals). Statistics between groups was performed on *Drosophila-Wolbachia* pairs with closest hub tropism frequencies between groups. Test for differences in proportions: between *D. sim* wRi and *D. tei* wTei, $P=0.025$; between *D. mau* wMau and *D. yak* wYak, $P=0.0008$. **L.** Quantification of *Wolbachia* density in the hub, normalized to the surrounding tissue (Error bars represent SEM for average density across all samples). Statistics between groups was performed on *Drosophila-Wolbachia* pairs with closest hub tropism densities between groups. T-test for differences in densities: between *D. sim* wRi and *D. sim* wNo, $P=0.027$; between *D. mau* wMau and *D. yak* wYak, $P<0.0001$. [For each host/*Wolbachia* pair, abbreviations are as follows: *D. ana* wAna, *Drosophila ananassae* infected with *Wolbachia ananassae*. See Supplemental Table 2.1 for details].

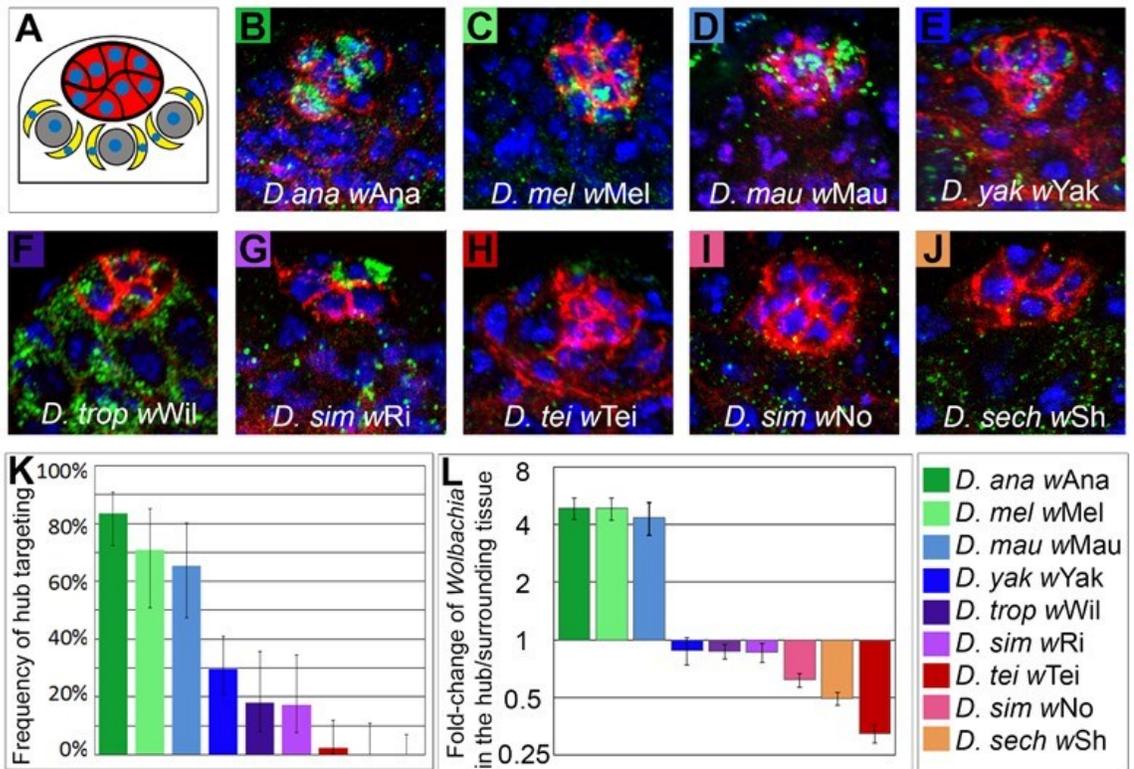


Figure 4.2: Comparison of evolutionary conservation of niche tropism in males and females

Diagrams of ovary and testis displaying *Wolbachia* tropism (green) to the GSCN and hub, respectively, are shown at the top. Ovary data adapted from (Toomey *et al.*, 2013). Color key at top right: Green= High hub/GSCN tropism, Yellow=moderate hub/GSCN tropism, Red=low/no hub/GSCN tropism (See supplemental table 3 for details). Pattern of *Wolbachia* tropism is evolutionarily conserved in the female ovary (A), but not in the testis (B). There is no clear correlation of tropism pattern with the *Wolbachia* phylogeny in the testis as was seen in the ovary ($p=0.773$).

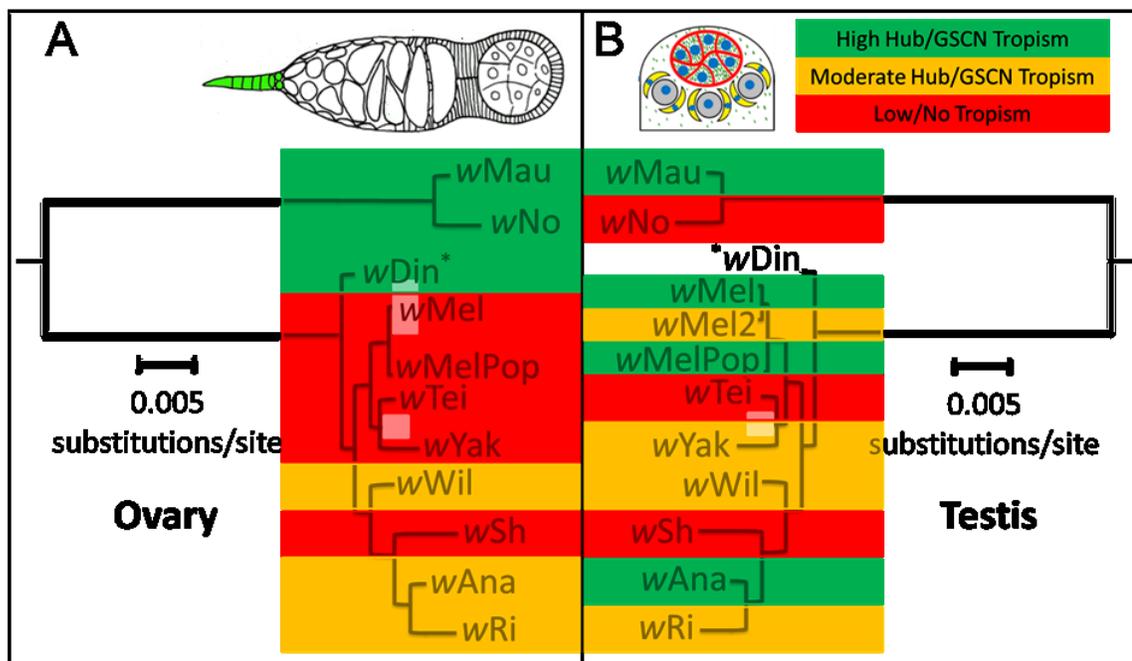


Figure 4.3: Random fit distribution of niche tropism on the *Wolbachia* phylogeny

A. Hub tropism phenotype traced and character fit to the phylogeny. *Wolbachia* phylogeny adapted from (Paraskevopoulos *et al.*, 2006). Hub tropism traced onto the *Wolbachia* phylogeny requires 6 steps. B. A set of 1000 random characters was computer simulated to assess the probability of the hub tropism character fit to the phylogeny due to chance. The probability of a fit as good, or better than the true character calculated for this phylogeny is 100%. Simulations performed with MacClade Software (Maddison *et al.*, 2005), see Chapter 2, Material and Methods.

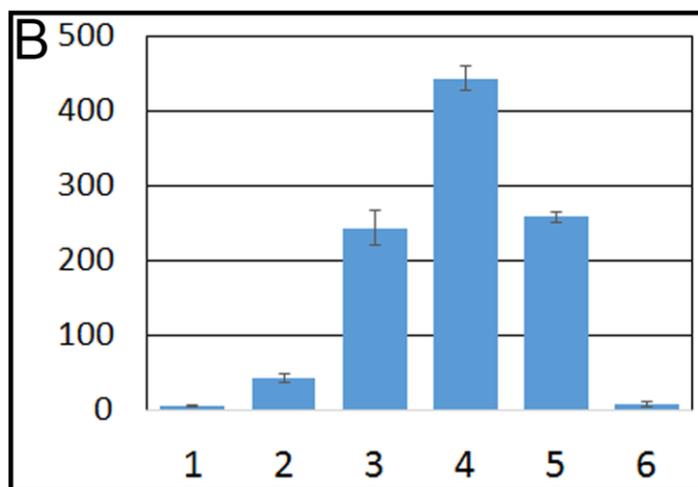
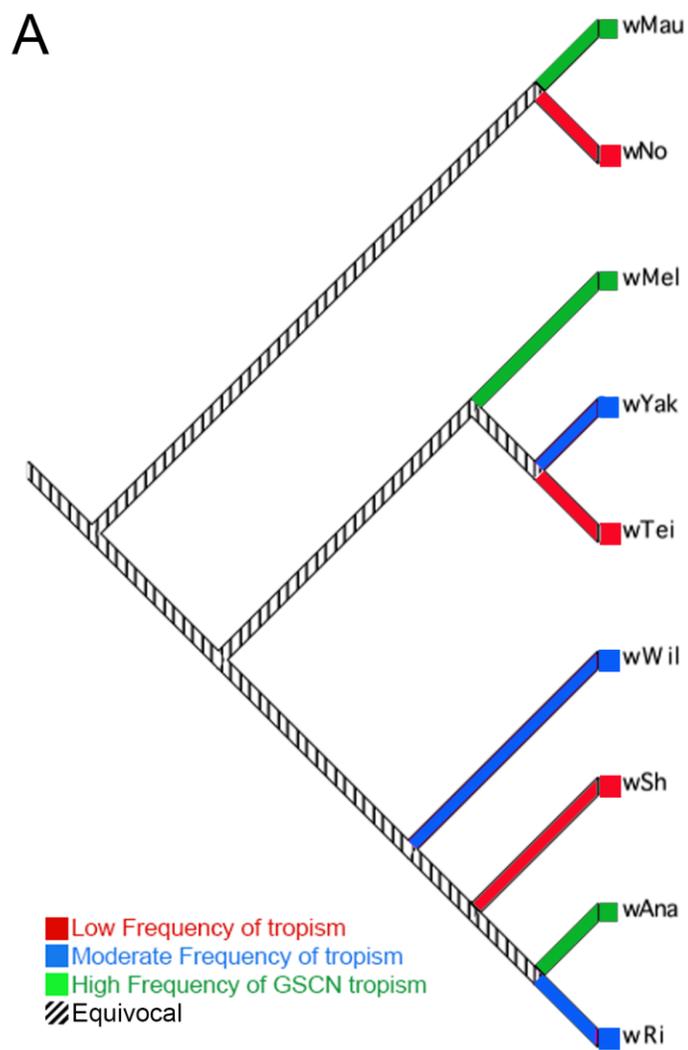


Figure 4.4: *Wolbachia* tropism to the hub does not correlate with either the *Drosophila* or *Wolbachia* phylogenies

Different patterns of niche targeting are correlated with *Drosophila* (left) and *Wolbachia* (right) phylogenies (phylogenies adapted from (Jefferis *et al.*, 1994; Paraskevopoulos *et al.*, 2006)) (MYA= million years ago). Green, blue, and red lines indicate high, moderate, and low frequency of hub tropism respectively. *wDin is a male killing strain of *Wolbachia*.

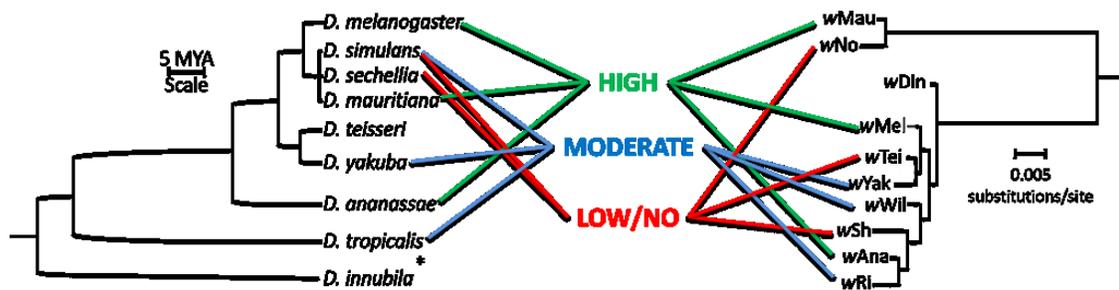


Figure 4.5: Random fit distribution of niche tropism on *Drosophila* phylogenies

A. Hub tropism phenotype traced to the *Drosophila* phylogeny (adapted from (Jeffs *et al.*, 1994)). Hub tropism traced onto the *Drosophila* phylogeny requires 5 steps. **B.** A set of 1000 random characters was computer simulated to assess the probability of the hub tropism character fit to the phylogeny due to chance. The probability of a fit as good, or better than the true character calculated for this phylogeny is 100%. Simulations performed with MacClade Software (Maddison *et al.*, 2005), see Chapter 2, Materials and Methods.

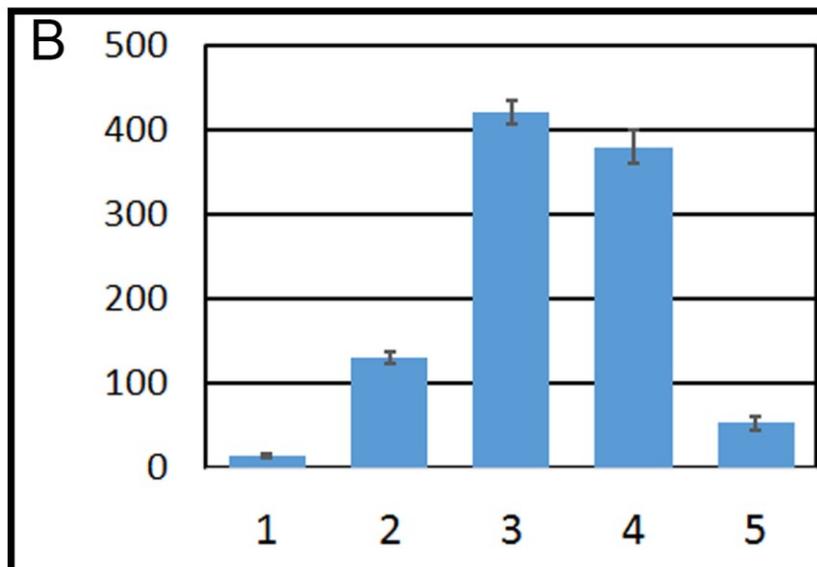
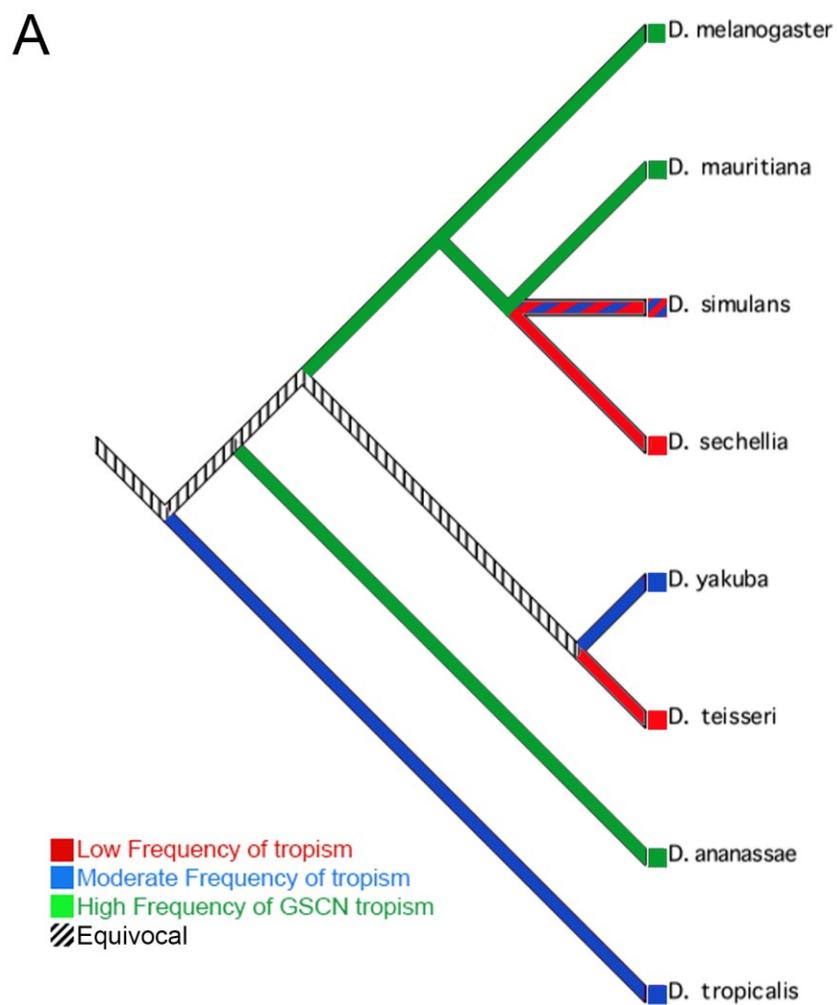


Figure 4.6: Both host and *Wolbachia* factors influence hub tropism

A. Representative images of *Wolbachia* tropism to the hub in parental *D. mauritiana* and *D. sechellia* testis (top row) and F₅ hybrid testis (bottom row) [*Wolbachia*, green; hub marker, red; DNA, blue]. Red and green arrows represent direction of *Wolbachia* transfer.

B. Quantification of frequency of hub tropism. Solid and hatched bars represent the parental and hybrid host species, respectively. Error bars represent 95% confidence intervals. Fisher Exact tests indicate that both the host genetic background and the *Wolbachia* strain have a significant effect on hub tropism ($p=8.309 \times 10^{-5}$ and $p=2.267 \times 10^{-10}$, respectively).

C. Quantification of *Wolbachia* density in the hub, normalized to the surrounding germline and soma. Linear regression analysis indicates that the *Wolbachia* strain ($P=0.045$), rather than the host genetic background ($P=0.56$), modulates *Wolbachia* density in the hub.

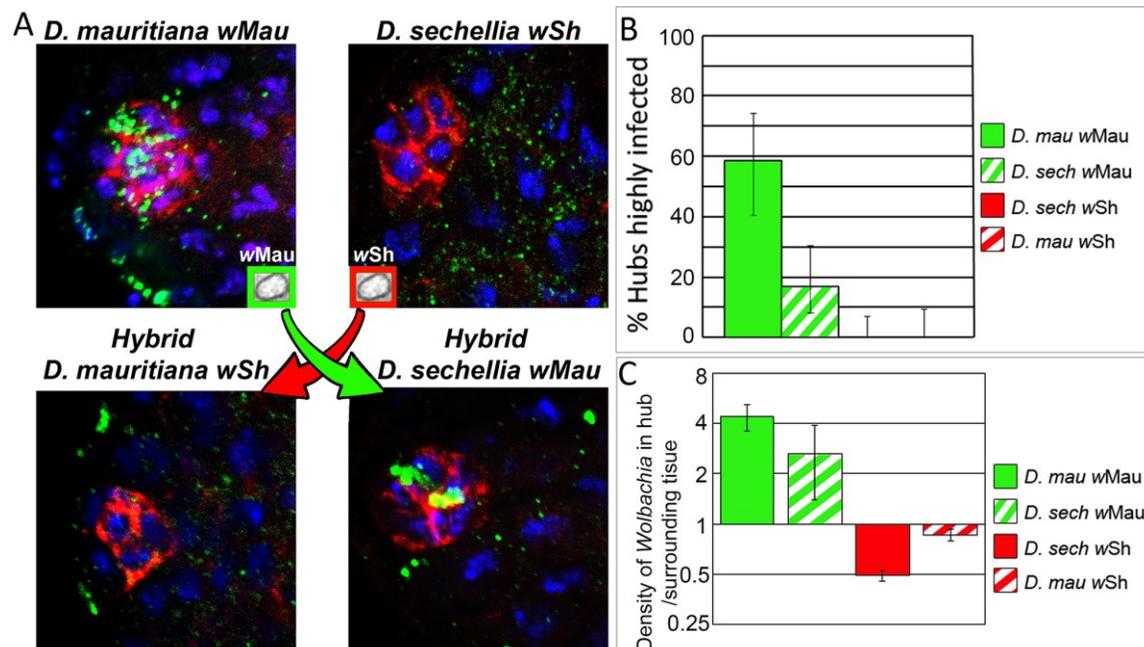


Figure 4.7: *Wolbachia* strain directs niche targeting in closely related *Drosophila* strains

A. Representative images of *Wolbachia* tropism to the hub in parental *D. simulans* strains 198 and 169 testis (top row) and F₅ hybrid testis (bottom row) [*Wolbachia*, green; hub marker, red; DNA, blue]. Red and green arrows represent direction of *Wolbachia* transfer.

B. Quantification of frequency of hub tropism. Solid and hatched bars represent the parental and hybrid host species, respectively. Logistical regression confirms *Wolbachia* factors have a significant effect on hub tropism ($P < 0.0001$) as compared to the host genetic background ($P = 0.927$).

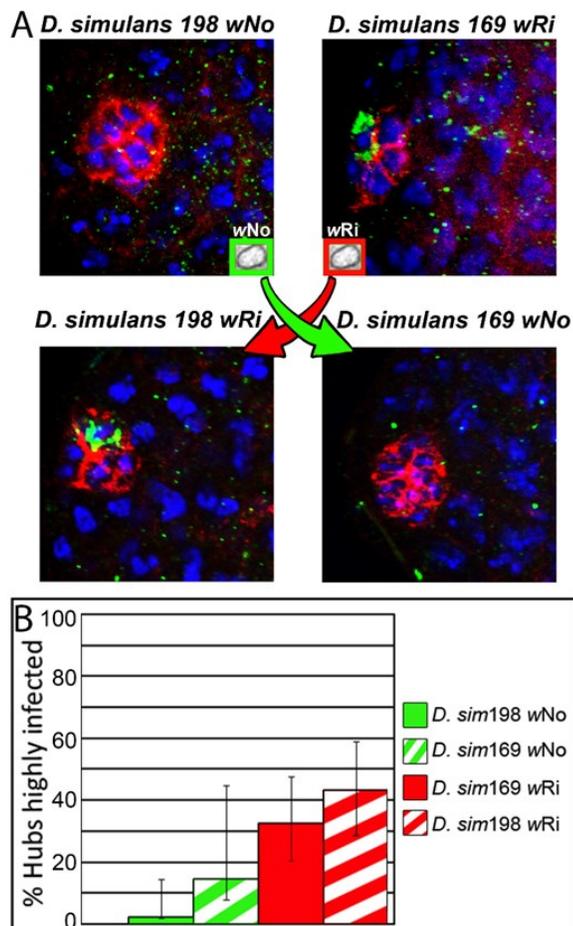


Figure 4.8: *Wolbachia* mediates niche tropism frequency and density in closely related bacterial strains

A-F. Representative images of *wMel*-like strains: *wMel*, *wMel2*, *wMel3* and *wMelCS*-like strains: *wMelCS2*, *wMelCS* and *wMelPop* infecting *D. melanogaster* hubs [*Wolbachia*, green; hub marker, red; DNA, blue]. **G.** Quantification of frequency of hub tropism. The three *wMel*-like *Wolbachia* strains target the hub at similar frequencies, significantly different from the *wMelCS*-like strains. (Frequencies with different letters are significantly different from one another, test for differences in proportions; Error bars represent 95% confidence intervals) **H.** Quantification of density of *Wolbachia* infecting the hub relative to the surrounding germline and soma. The three *wMel*-like *Wolbachia* strains target the hub at similar densities, and are significantly different from the *wMelCS*-like strains. (Means with different letters are significantly different from one another as determined by a t-test; Error bars represent SEM).

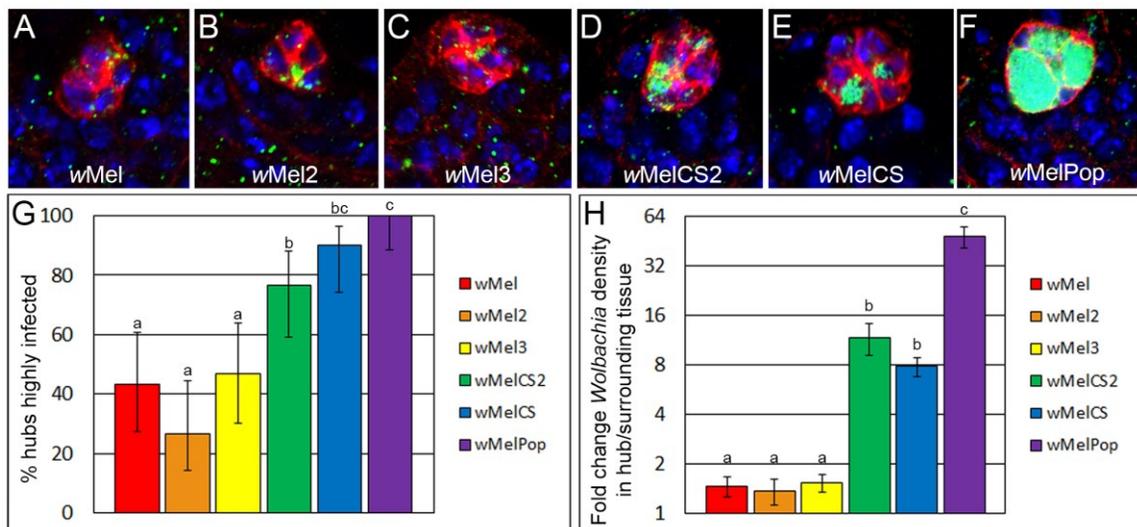


Figure 4.9: Hubs infected with *w*MelPop burst open

A-C. Representative images of hubs classified as normal high niche infection (HN, **A**), abnormal hub morphology suggestive of swelling, but not yet bursting (**B**), and bursting (**C**). *Wolbachia* is stained in green and the hub is in red. **A'-C'** insets of each image show the gray scale of the *Wolbachia* channel. **A''-C''** insets of each image show the gray scale of the hub marker. In the bursting hub (**C''**), it is evident that the hub cell membrane has been broken open. **D.** Quantification of hub infection phenotype. Scale Bar is 5 μ m.

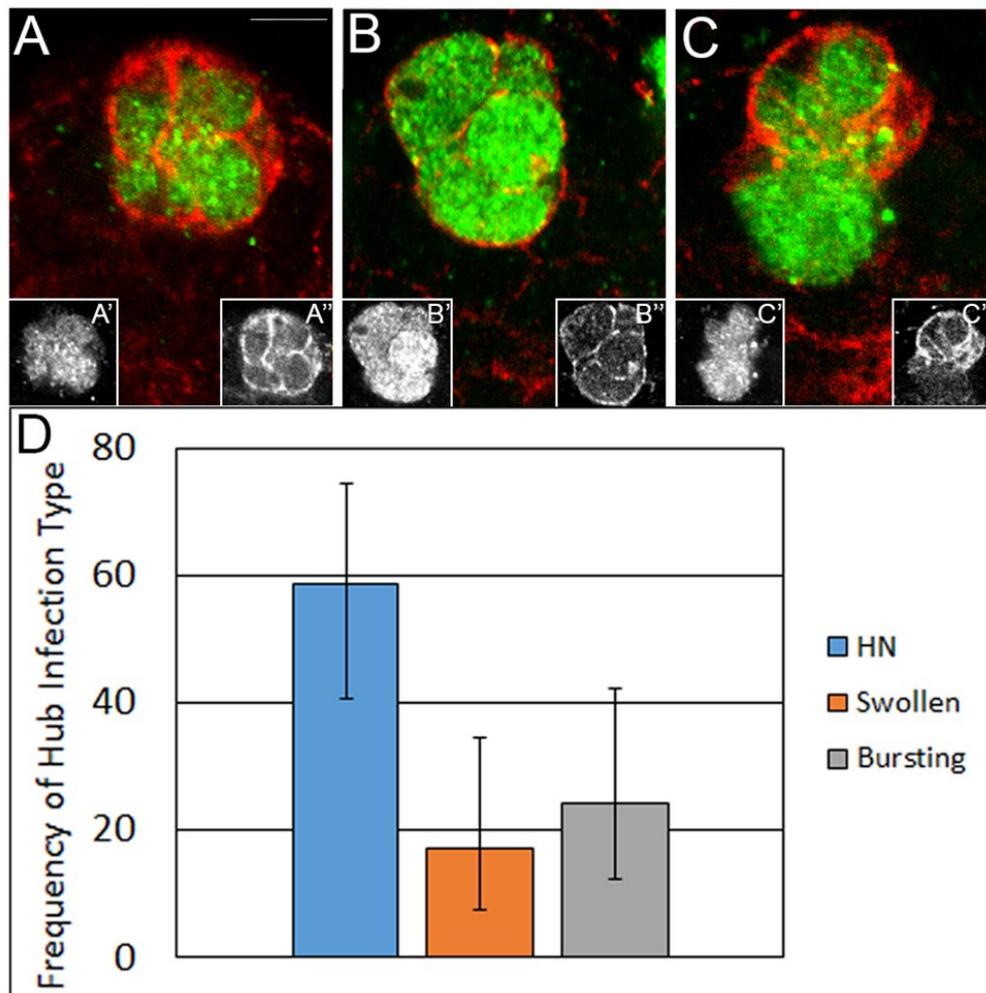


Table 4.1: Frequencies and densities of *Wolbachia* hub tropism in diverse *Drosophila-Wolbachia* pairs

Species	<i>Wolbachia</i> strain	N	Frequency	Density
<i>D. sechellia</i>	wSh	51	0.00%	0.32
<i>D. simulans</i>	wNo	31	0.00%	0.49
<i>D. teissieri</i>	wTei	43	2.33%	0.62
<i>D. simulans</i>	wRi	29	17.24%	0.87
<i>D. tropicalis</i>	wWil	28	17.86%	0.86
<i>D. yakuba</i>	wYak	74	29.73%	0.88
<i>D. mauritiana</i>	wMau	29	65.52%	4.38
<i>D. melanogaster</i>	wMel	24	70.83%	4.90
<i>D. ananassae</i>	wAna	61	83.61%	6.32

Tropism for the hub was quantified using MatLab imaging software and confocal imaging. For each individual fly, *Wolbachia* infection of the hub was qualified as “hub tropism” if the density was at least 1.5-fold higher in the hub than the surrounding tissue. Frequency shows the percent of flies that satisfied this criterion. The overall density of the species is shown.

Table 4.2: Hub tropism does not correlate with GSCN tropism in the ovary

<i>Drosophila</i> Species	<i>Wolbachia</i> Strain	Frequency of Hub Tropism	Frequency of GSCN Ovary Tropism*
<i>D. sechellia</i>	wSh	0.00	0.83
<i>D. simulans</i>	wNo	0.00	99.28
<i>D. teissieri</i>	wTei	2.33	3.64
<i>D. simulans</i>	wRi	17.24	53.54
<i>D. tropicalis</i>	wWil	17.86	32.73
<i>D. yakuba</i>	wYak	29.73	0.97
<i>D. mauritiana</i>	wMau	65.52	96.00
<i>D. melanogaster</i>	wMel	70.83	0.96
<i>D. ananassae</i>	wAna	83.61	51.09

The presence or absence of stem cell niche tropism in males was compared to previously determined tropism in the female GSCN *(Toomey *et al.*, 2013). Frequencies from 0-9% are considered low/no tropism; 10-59% are considered moderate tropism; 60-100% are considered high tropism. Statistical correlation test shows no relationship between males and females (P=0.773).

Table 4.3: Hub tropism does not correlate with cytoplasmic incompatibility

<i>Wolbachia</i> Strain	Frequency of Hub Tropism (%)	Levels of CI
wSh	0.00	high (Charlat <i>et al.</i> , 2002)
wNo	0.00	mod (Charlat <i>et al.</i> , 2002; Zabalou <i>et al.</i> , 2004)
wTei	2.33	low (Zabalou <i>et al.</i> , 2004)
wRi	17.24	high (Van Meer <i>et al.</i> , 1999)
wWil	17.86	unknown
wYak	29.73	low (Zabalou <i>et al.</i> , 2004)
wMau	65.52	low (Veneti <i>et al.</i> , 2003)
wMel	70.83	low (Van Meer <i>et al.</i> , 1999)
wAna	83.61	mod (Bourtzis <i>et al.</i> , 1996)
wMelPop	94.52	low (Van Meer <i>et al.</i> , 1999; Veneti <i>et al.</i> , 2003)

(Correlation test, P=0.267).

Table 4.4: Frequency of *Wolbachia* targeting in *D. mauritiana*, *D. sechellia*, and the hybrid backcrossed F5 progeny

Species	<i>Wolbachia</i> strain	N	%
<i>D. mauritiana</i>	wMau	29	65.52%
<i>D. sechellia</i>	wMau	42	16.67%
<i>D. sechellia</i>	wSh	51	0.00%
<i>D. mauritiana</i>	wSh	27	0.00%

Hybrid lines bolded.

Table 4.5: Frequency of *Wolbachia* targeting in *D. simulans* and the hybrid backcrossed F5 progeny

Species	<i>Wolbachia</i> strain	N	%
<i>D. simulans</i> 169	wRi	43	32.56%
<i>D. simulans</i> 198	wRi	37	43.24%
<i>D. simulans</i> 198	wNo	43	2.33%
<i>D. simulans</i> 169	wNo	34	14.71%

Hybrid lines bolded.

Table 4.6: Frequencies and densities of *Wolbachia* hub tropism in *D. melanogaster*

<i>Wolbachia</i> Strain	N	HN	Frequency	Density
wMel	30	13	43.33	1.46
wMel2	30	8	26.67	1.37
wMel3	30	14	46.67	1.54
wMelCS2	30	23	76.67	11.67
wMelCS	30	27	90.00	7.81
wMelPop	30	30	100.00	47.99

Tropism for the hub was quantified using MatLab software and confocal imaging. *Wolbachia* infection of the hub was considered tropism if the density was 1.5-fold higher in the hub than the surrounding tissue.

CHAPTER 5

Cellular and Molecular Mechanisms of *Wolbachia* Hub Tropism

5.1 Introduction

Tissue tropism is an essential aspect of microbial-host interactions. The tissues preferentially infected by the microbe can determine the consequences of infection for the host as well as the successful establishment of infection for the microbe. *Wolbachia* has been widely shown to have a tropism for the female host germline. This tropism is essential for *Wolbachia* propagation due to its maternal mode of transmission through the egg cytoplasm. Even in cases where *Wolbachia* are horizontally transmitted, in order to become established in a population, *Wolbachia* must colonize the germline to be successfully maternally transmitted. Although *Wolbachia* is mainly vertically transmitted, infection throughout the host is widespread and somatic tissues are often targeted (Hosokawa *et al.*, 2010; Landmann *et al.*, 2010). The cellular and molecular mechanisms involved in *Wolbachia* tropism to different tissues within the host are poorly understood, in part because genetic manipulation of *Wolbachia* is currently not possible.

Previous work in our lab has revealed a peculiar tropism of *Wolbachia* to the stem cell niches in the *Drosophila* gonads. In the ovary, this tropism has been shown to facilitate vertical transmission as well as modulate stem cell activity (Fast *et al.*, 2011; Toomey *et al.*, 2013). *Wolbachia* tropism to the stem cell niches in *Drosophila* allows the use of a wide array of genetic and molecular tools in a very well characterized model organism. However, molecular characterization of stem cell niche tropism in the *D. mel* ovary is challenging. In the *D. mel* ovary, *Wolbachia* target the SSCN. Compared to the GSCN, the

SSCN is less well characterized, with few tools available for manipulation. Furthermore, *Wolbachia* strains that target the GSCN in the ovary infect *Drosophila* species that have few genetic tools available.

The *D. mel* testis stem cell niche, the hub, offers a unique system to investigate the mechanisms involved in tropism to stem cell niches. *wMel* and *wMelCS* both display tropism to the hub, and accumulate at significantly different densities. The hub is a developmentally, cellularly, and molecularly well characterized structure. It is formed from a subset of somatic gonadal precursors (SGPs) which are specified prior to stage 12 of embryogenesis. The anterior subset of SGPs expressing *escargot*, *fasciclin 3*, *Drosophila E-cadherin (DE-cad)*, and *unpaired (upd)* form the hub during stage 17 of embryogenesis. These SGPs segregate into a cluster in a distinct region of the gonad, surrounded by a subset of germ cells. The SGP cluster is reminiscent of the rosette morphology of the adult hub (Le Bras *et al.*, 2006).

The adult hub is a group of 10 to 16 cells located at the tip of the testis lobe, forming the microenvironment supporting the adjacent germline and cyst stem cells, referred to as the niche (Hardy *et al.*, 1979). We took advantage of the tools available in the *D. mel* hub to dissect the cellular and molecular mechanisms of *Wolbachia* tropism in this system. Determining the cellular and molecular bases of tissue tropism is essential to understanding *Wolbachia*-host interactions.

5.2 *Wolbachia* accumulate in the hub throughout development

Wolbachia tropism to the hub was characterized throughout development to gain insight into the cellular mechanisms of accumulation. L3 larval, newly eclosed (NE) adult,

and 7 day (7d) old adult testes were dissected and immunostained to quantify the frequency of *Wolbachia* hub tropism and the density of *Wolbachia* in the hub throughout development (Figure 5.1, Table 5.1, $N \approx 30$ hubs for each sample).

By the L3 stage, over 90% of all hubs were infected with *Wolbachia* for both *wMel* and *wMelCS* infected flies (Figure 5.1G), with no significant difference in frequency from L3 to 7d for *wMel* or *wMelCS* (Chi-square; $P_{\text{mel}}=0.167$ and $P_{\text{cs}}=0.218$). The density of *Wolbachia* within the hub, however was lowest in hubs of L3 larvae for both *wMel* and *wMelCS*. The densities significantly increased from L3 to NE to 7d for *wMel* (Figure 5.1H; t-test: $P_{\text{L3-NE}}=0.011$; $P_{\text{NE-7d}}=0.012$) and from NE to 7d for *wMelCS* (Figure 5.1H; t-test: $P < 0.0001$). These results indicate that *Wolbachia* target the hub at an early developmental stage and accumulate to higher densities over time.

5.3 Mechanisms of hub tropism

5.3.1 Cellular mechanisms of accumulation

Data from the developmental profile of *Wolbachia* hub tropism suggest that *Wolbachia* first target the hub early in development, then preferentially replicate within the hub cells as compared to the surrounding germline and soma. To experimentally test this model, we conducted two experiments: 1) we measured the fraction of dividing *Wolbachia* in the hub as compared to the surrounding germline and soma; and 2) we blocked *Wolbachia* replication with a bacteriostatic antibiotic and measured densities of *Wolbachia* in the hub.

An alternative model could be preferential invasion of the hub cells from the surrounding tissue. This is difficult to directly test, given that the mechanism for cell

invasion is currently unknown. Interestingly, the gram positive, intracellular bacteria *Listeria monocytogenes* utilizes an E-cadherin mediated mechanism to invade cells. A *Listeria* extracellular membrane protein, Internalin A, specifically recognizes E-cadherin on the surface of the host cell, inducing cytoskeletal rearrangements via α - and β - catenin, and culminating in bacterial uptake (Mengaud *et al.*, 1996; Lecuit *et al.*, 2000; Hamon *et al.*, 2006). All of these proteins are highly expressed on the hub cell surface, making this a plausible mechanism of *Wolbachia* entry into hub cells.

5.3.1.1 *Wolbachia* preferentially replicate within the hub, relative to surrounding germline and soma

Towards elucidating the cellular mechanism of *Wolbachia* accumulation in the hub during development, the fraction of dividing *Wolbachia* inside the hub versus the surrounding germline and soma was compared. If the mechanism of *Wolbachia* hub accumulation is preferential replication, then we would expect to see a higher fraction of dividing *Wolbachia* inside the hub relative to the surrounding tissue. Localization of the highly conserved bacterial fission protein, FtsZ, was used to determine if a single *Wolbachia* was actively dividing. In actively dividing bacteria, FtsZ is localized to the septation ring (Figure 5.2A). When not dividing, FtsZ is tethered to the membrane in a structure, possibly through ZipA or FtsA in a helical pattern along the length of the bacterium (Figure 5.2B) (Margolin, 2005). Since single *Wolbachia* need to be analyzed, this analysis could only be conducted with *w*Mel infected flies, upon eclosion, where *Wolbachia* densities are relatively lower (as compared to newly eclosed *w*MelCS infected hubs). It was also important to conduct this analysis during a developmental time when the

Wolbachia density is increasing significantly (i.e., from NE to 7d in *wMel*, see Figure 5.1H).

The total number of discernable dividing and non-dividing *Wolbachia* inside the hub and in the surrounding germline and soma were counted (Figure 5.2C, Table 5.2). Figure 5.3D displays a representative image with a dividing *Wolbachia* inside the hub, a non-dividing *Wolbachia* outside the hub, and a clump of uncountable *Wolbachia* inside the hub. Supporting our model of preferential replication, there is a significantly higher fraction of dividing *Wolbachia* inside the hub as compared to the germline and soma surrounding the hub (Figure 5.2E; t-test $P < 0.0001$).

5.3.1.2 Inhibition of *Wolbachia* replication supports preferential replication model of *Wolbachia* accumulation in the hub

To further validate the model of preferential replication, a bacteriostatic antibiotic was used to block *Wolbachia* replication during development. Tetracycline is a bacteriostatic antibiotic that acts by inhibiting protein synthesis. It binds to the 30S subunit of microbial ribosomes and blocks the attachment of tRNAs (Maxwell, 1967; Brodersen *et al.*, 2000). This process is reversible by removal of tetracycline. To block replication of *Wolbachia* inside the flies during development, a sub-optimal dose of tetracycline (0.05 mg/mL) was fed to the larvae and/or adult flies, depending on the experimental sample (as outlined in Figure 2.2). Adult flies were analyzed upon eclosion, treated vs. non-treated, or at 7 days old, treated continuously from larvae through adulthood, or removed from tetracycline upon eclosion and allowed to recover (for schematic see Figure 2.3). *Wolbachia* densities were then measured in the hub relative to the surrounding tissue.

Overall, the results demonstrate that *Wolbachia* accumulation ($wMel$ and $wMelCS$) in the hub can be reduced when the larvae are fed tetracycline throughout development (Figures 5.3 and 5.4, Table 5.3).

When flies were raised on tetracycline from the 1st instar larvae, the frequency of hub targeting in newly eclosed flies was significantly reduced for both $wMel$ and $wMelCS$ as compared to untreated flies (Figure 5.3F: $wMel_{NE,Tet}=61.5\%$, $wMel_{NE,NoTet}=82\%$, Test for differences in proportions $P=0.022$; Figure 5.4F: $wMelCS_{NE,Tet}=52\%$, $wMelCS_{NE,NoTet}=85\%$, Test for differences in proportions $P=0.002$). Interestingly, when adults were removed from tetracycline treated food and aged on normal fly food, *Wolbachia* densities significantly recovered (t-test: $wMel_{NE,Tet \rightarrow 7dTet-NoTet}$ $P=0.0022$; $wMelCS_{NE,Tet \rightarrow 7dTet-NoTet}$ $P<0.0001$), but the frequencies of infected hubs remained unchanged (Test for differences in proportions: $P>0.05$). This suggests that the accumulation of *Wolbachia* in the hub is not due to *Wolbachia* invasion from the surrounding tissue (since frequency of targeting would also recover when flies are removed from tetracycline), but to *Wolbachia* replication within the hub cells.

5.3.2 Molecular mechanism of accumulation

Although *Wolbachia* cannot be genetically manipulated, *D. mel* offers an array of genetic and molecular tools to target host proteins for knockdown or overexpression in order to assess their effect on *Wolbachia*. We utilized a candidate gene approach to identify host proteins or signaling pathways that affect *Wolbachia* densities within the hub. Candidate pathways for *Wolbachia* entry, as well as candidate genes that could be modulating *Wolbachia* density within the hub were analyzed. There are several signaling

pathways and proteins that are highly expressed in the hub (Figure 1.4). Unpaired, the ligand for JAK-STAT signaling, is specifically expressed in the hub and is secreted to the adjacent stem cells to control their self-renewal. Adherens junctions, composed of the transmembrane DE-Cadherin and α - and β - catenins, are also highly expressed and important for both hub cell to hub cell adhesion as well as stem cell adhesion to the hub (Matunis *et al.*, 2012). These proteins are also highly expressed in other tissues where *Wolbachia* infect at high densities, including the GSCN and SSCN (Song *et al.*, 2002; Song *et al.*, 2002; Toomey *et al.*, 2013), and polar cells (Ajit Kamath, unpublished data and (Peifer *et al.*, 1993)) in the ovaries. Therefore, we began our candidate gene approach by investigating whether any of these proteins involved in stem cell biology were involved with *Wolbachia* tropism in the hub.

5.3.3.1 Unpaired levels do not influence *Wolbachia* levels in the hub

Unpaired is the ligand for the JAK-STAT pathway in *Drosophila*, which is expressed in several cell types in the fly, including the stem cell niches in the ovaries and testes (Decotto *et al.*, 2005), as well as the polar cells in the ovaries (Silver *et al.*, 2005). All of these cell types also accumulate *Wolbachia* at high densities. To investigate if *upd* expression is a molecular cue for *Wolbachia* accumulation in the hub, we knocked down *upd* using RNAi as well as overexpressed *upd* specifically in the hub using the GAL4-UAS system. Knocking down *upd* in the hub resulted in no significant differences in *Wolbachia* tropism frequency or density in newly eclosed flies, as compared to sibling control flies lacking the RNAi construct (Figure 5.5A, Table 5.4). Similarly, overexpression of *upd* in the hub also resulted in no significant differences in *Wolbachia* tropism in newly eclosed

or 7 day old flies (Figure 5.5 B and C, Table 5.5). qPCR of *upd* mRNA levels in whole testis expressing RNAi or overexpression of *upd* in the hub (compared to control flies) indicated a modest reduction (30-60%) and increase (30-60%) in *upd* levels, respectively (Figure 5.6). The lack of significant difference in the levels of *Wolbachia* could be due to the inadequate reduction or overexpression of the Upd (protein) levels in the hub, however, these results suggest that perturbations in *upd* mRNA levels do not significantly alter *Wolbachia* tropism in the hub.

5.3.3.2 Adherens junctions and Wnt/Wg signaling

Armadillo (Arm, *Drosophila* homolog of β -catenin) is a protein involved in Wnt (also known as Wingless) signaling as well as adherens junctions. Arm is highly expressed on the hub cell surface and is widely used as a marker for this structure. In Wnt signaling, the Wnt signal is transduced by Frizzled receptors, leading to the phosphorylation of Disheveled, the inhibition of Shaggy and Axin, and the stabilization of Arm. Arm then translocates into the nucleus where it activates target gene expression with its partner Pangolin (Bejsovec, 2013). Wnt signaling is involved in regulating many processes during *Drosophila* development and the Wnt ligand, Wingless, is transcribed in CySCs and early cyst cells in the testis (Leatherman *et al.*, 2008; Pancratov *et al.*, 2013). Although Wnt signaling is known to be involved in stem cell maintenance in the ovaries (Song *et al.*, 2003), the involvement of Wnt signaling in testis stem cell biology has not been described. Armadillo, as a member of adherens junctions, is directly involved in stem cell maintenance by anchoring the stem cells to the niche via DE-Cadherin. Given its

implications in hub biology, Arm was investigated for its effect on *Wolbachia* tropism to the hub.

5.3.3.2.1 Knockdown of Armadillo in the hub results in reduced *Wolbachia* densities

Using the GAL4-UAS system, we drove expression of RNAi against *arm* in the hub using the *upd-GAL4* driver. *Wolbachia* densities were measured in the hub and normalized to the surrounding germline and soma densities. The relative density of *Wolbachia* in the hub was compared between hubs with robust RNAi mediated knockdown of *arm* and sibling control flies with only the driver (lacking the *UAS-RNAi* construct). In control hubs, Arm accumulates in adherens junctions on the hub cell surface (Figure 5.7A), and can be used as a hub marker due to its robust immunostaining. Therefore, immunostaining against Arm can be used to assess the efficiency of *arm* knockdown in the hub. Only hubs with no detectable Arm staining were considered for *Wolbachia* density (Figure 5.7B). We found a significant reduction of *Wolbachia* densities in hubs with robust knockdown of *arm* for both *wMel* and *wMelCS* infected hubs (Figure 5.7, *wMel*: 4-fold, $P < 0.0001$; *wMelCS*: 3-fold, $P = 0.022$, Table 5.6). These data indicate that a reduction of Arm levels results in a reduction of *Wolbachia* levels in the hub.

5.3.3.2.2 Armadillo does not modulate *Wolbachia* levels via adherens junctions

Extensive literature has shown that the major function of Arm in the hub is anchoring the adjacent stem cells via adherens junctions (Song *et al.*, 2002; Song *et al.*, 2002; Leatherman *et al.*, 2010). To investigate if the Armadillo dependent decrease in *Wolbachia* levels in the hub was through adherens junctions, we targeted *Drosophila* E-

Cadherin (DEC) for RNAi-mediated knockdown in the hub using the *upd-GAL4* driver. Similarly to Arm, in control hubs, DEC accumulates in adherens junctions on the hub cell surface (Figure 5.9A), and can be used as a hub marker due to its robust immunostaining. Immunostaining against DEC was used to assess the efficiency of *DEC* knockdown in the hub. Only hubs with no detectable DEC staining were considered for *Wolbachia* density (Figure 5.9B). We found no significant reduction of *Wolbachia* densities (*wMel* or *wMelCS*) in *DEC-RNAi* expressing hubs as compared to sibling control flies lacking the *UAS-RNAi* construct (Figure 5.10, Table 5.7). Therefore, the reduction in *Wolbachia* densities in *arm-RNAi* hubs is not through adherens junctions.

5.3.3.2.3 Armadillo levels may affect *Wolbachia* levels through Wnt/Wg signaling

Apart from its well described function in cell adhesion for stem cell maintenance, Arm may also regulate Wnt signaling and transcription of Wnt target genes in the hub. Although little is known about the contributions of Wnt signaling to stem cell maintenance in the testis, it has been shown to directly regulate somatic stem cell (SSC) maintenance in the ovary. Constitutive Wnt signaling in the ovary causes over proliferation and improper differentiation of daughter follicle cell progeny and reduction in Wnt signaling induces SSC loss (Song *et al.*, 2003). *wg* is expressed in the somatic cells close to the hub, including the Cyst Stem Cells (CySCs) and early cyst cells (Leatherman *et al.*, 2008; Pancratov *et al.*, 2013). Furthermore, it has been shown that when CySCs are genetically ablated, hub cells exit quiescence and enter the cell cycle to replace the ablated CySCs. These hub cells show reduced levels of Armadillo, suggesting that they delaminate from the hub as they

exit quiescence (Hetie *et al.*, 2014). Although the literature describing Wnt signaling in the hub is limited, we investigated whether Armadillo could be affecting *Wolbachia* levels in the hub through its role in Wnt signaling by expressing an *armadillo* construct that is constitutively active in Wnt signaling (*arm^{S10}*). The *arm^{S10}* mutant construct has a 54 amino acid deletion in the N-terminal domain, and is missing a GSK/Zw3 phosphorylation site, as well as an ubiquitination site, allowing for the escape from normal negative regulation. This mutant form of *arm* is degradation resistant, allowing for accumulation of cytosolic Arm resulting in the activation of the Wnt pathway in the absence of the Wnt signal, and is also active in adherens junctions. In embryos, when activated by e22c-GAL4 (somatic cell driver), Arm^{S10} levels accumulate to roughly those of WT endogenous Arm, increasing overall Arm levels 2.4-fold (Pai *et al.*, 1997).

Using the *GAL4-UAS* system, we expressed *arm^{S10}* using the *upd-GAL4* driver to assess if increasing Wnt signaling would result in increased *Wolbachia* levels. Because the combination of driver and construct was male pupal lethal, we assessed *Wolbachia* levels in L3 larval hubs of flies expressing the *UAS-arm^{S10}* construct and control flies lacking the construct. For *wMel* and *wMelCS* infected hubs, there were 2.1- and 2.9-fold increases in *Wolbachia* density, respectively, in hubs expressing *arm^{S10}* as compared to control hubs (Figure 5.11, statistically significant, t-test: $P_{wMel}=0.0046$, $P_{wMelCS}=0.0007$, Table 5.8). These results suggest that *Wolbachia* densities in the hub are modulated by Arm via Wnt signaling.

5.3.3.2.6 Armadillo levels also affect *Wolbachia* levels in polar cells in the *Drosophila* ovary

To determine whether *Wolbachia* modulation by Arm also occurs in other cell types, in collaboration with Ajit Kamath, we investigated if altering Armadillo levels in another cell type affected *Wolbachia*. In the *D. mel* ovary, *Wolbachia* highly colonize the somatic stem cell niche. Because there are few molecular tools available to genetically manipulate this structure, we sought to investigate another cell type in the ovary harboring high levels of *Wolbachia*, the polar cells (PCs). The PCs are a group of 2-3 cells located at either pole of each egg chamber important for the patterning and development of the oocyte. Similar to the other follicle cells (FCs), the PCs are somatically derived from the asymmetric division of the somatic stem cell (SSC) and are specified in stage 2 of oogenesis. We found that *Wolbachia* highly accumulate in these cells as compared to the surrounding follicles cells (Ajit Kamath, unpublished data).

Similarly to our approach in the hub, we expressed RNAi against *arm* using the *GAL4-UAS* system in the polar cells and measured *Wolbachia* levels in PCs (relative to the surrounding FCs) with and without the RNAi construct. We found a significant reduction in *Wolbachia* levels in PCs when *arm* was knockdown, as compared to sibling control flies lacking the RNAi construct for both *wMel* and *wMelCS* infected flies (Figure 5.12, 3-fold and 2-fold change, respectively). These results demonstrate that Arm modulates intracellular *Wolbachia* densities in multiple cell types.

5.4 Discussion

Microbial tissue tropism is a fundamental aspect of host-microbe interactions which dictates the infection sites of the microbe in the host. *Wolbachia* has a selective tropism to the stem cell niches in the ovaries and testes, where it can manipulate stem cell biology (Fast *et al.*, 2011) as well as facilitate vertical transmission (Toomey *et al.*, 2013). Although stem cell niche tropism has been well characterized at the cytological level across the *Drosophila* genus, the molecular mechanisms contributing to stem cell niche tropism in *Drosophila* gonads are unknown.

Using *Wolbachia* tropism to the hub in *D. mel* as our model, we investigated the developmental kinetics of infection, as well as the cellular and molecular mechanisms of stem cell niche tropism. We found that *Wolbachia* targets the hub early in development, likely during hub specification in stage 17 of embryogenesis, and preferentially replicates within this structure to achieve high densities throughout the development of the host. Through a candidate screen we found that Arm levels within the hub affect intracellular *Wolbachia* titers. RNAi-mediated knockdown of *arm* in the hub using the *GAL4-UAS* system resulted in decreased *Wolbachia* titers in this structure. Towards identifying a mechanism for how Arm modulates *Wolbachia* levels, we ruled out the role of Arm in adherens junctions. Furthermore, overexpression of a constitutively active form of Arm indicates that by increasing Wnt signaling in the hub, *Wolbachia* titers increase. Finally, we show that modulation of *Wolbachia* densities through Arm is conserved in another cell type in the ovary with high titers of *Wolbachia*, the polar cells. These data provide a

foundation to investigate the mechanisms of tropism to many other cell/tissue types in diverse hosts.

Based on the developmental kinetics of hub infection, we propose two models for how *Wolbachia* achieve high densities in the hub: preferential replication or preferential invasion. Several lines of evidence support preferential replication as our cellular mechanism for hub tropism. Blocking replication of *Wolbachia* during development using a bacteriostatic antibiotic (tetracycline), then allowing for the recovery over 7 days, further revealed that *Wolbachia* intracellular accumulation can be blocked, and then recovered. However, the frequency of infected hubs did not recover upon the removal of antibiotics. These data indicate that *Wolbachia* do not invade the hub during adulthood, and therefore, preferential invasion of the hub cells cannot account for the increase in *Wolbachia* density observed in the developmental kinetics experiments. Furthermore, direct measurement of the fraction of dividing *Wolbachia* indicated that *Wolbachia* are more frequently divide in the hub as compared to the surrounding soma and germline. The preferential replication model is further supported by data of GSCN tropism in the ovary, where *Wolbachia* also more frequently divide in the niche relative to the surrounding soma (Eva Fast, unpublished data).

We next sought to unravel the molecular mechanisms involved in *Wolbachia* hub tropism. We approached this question by targeting host proteins enriched in the hub cells for involvement in *Wolbachia* tropism. A targeted screen for RNAi-mediated knockdown of proteins enriched in the hub revealed a role for Armadillo in *Wolbachia* hub tropism. Upon RNAi-mediated knockdown of *arm*, *Wolbachia* levels in the hub decreased. This

interaction could be directly related to Arm levels, or indirect through several different pathways Arm is involved in, such as adherens junctions, Wnt signaling, or other non-canonical pathways.

To further investigate Arm's role in *Wolbachia* hub tropism, we investigated two pathways that Arm is involved in for their potential contribution to *Wolbachia* hub tropism: adherens junctions and Wnt signaling. Knockdown of DE-Cadherin, another protein component of adherens junctions, ruled out adherens junctions as the mechanism modulating *Wolbachia* levels in the hub. Expression of a constitutively active *arm* (*arm^{s10}*), led to an increase in *Wolbachia* levels in the hub, implicating Wnt signaling as the potential mechanism for the modulation of *Wolbachia* levels in the hub. Although further experiments are necessary to reveal the exact mechanism of *Wolbachia* hub tropism, the data support a model in which Wnt signaling is involved.

Wnt signaling is involved in the regulation of many processes during *Drosophila* development, most notably embryo segmentation and polarity. In the testis, Wnt is transcribed in CySCs and early cyst cells (Leatherman *et al.*, 2008; Pancratov *et al.*, 2013). Although much is known about stem cell maintenance in the *Drosophila* testis, little is known about the contribution of Wnt signaling to this process. It is possible that the Wnt signal from the surrounding CySCs is activating the Wnt signaling cascade in the hub. A downstream effect of activating Wnt in the hub could be that induction of Wnt-responsive genes promotes *Wolbachia* replication in this structure. Recent data have shown that when CySCs are genetically ablated, there is a reduction/loss of Arm in some hub cells, which then exit quiescence and replace the CySCs that were lost (Hetie *et al.*, 2014). In intact

niches, extrinsic signals from the CySCs may contribute to the prevention of hub cell conversion. It is possible that this is the role Wnt signaling normally plays in the testis stem cell niche, and *Wolbachia* replication to the hub is a consequence of normal Wnt signaling in this structure.

There is substantial literature investigating intracellular factors/pathways which promote *Wolbachia* density, however, none have directly implicated *wnt* signaling. Pathways implicated include autophagy, proteasomal degradation, and microRNAs (Fallon *et al.*, 2009; Voronin *et al.*, 2012; Zhang *et al.*, 2013). It has been reported that autophagy regulates *Wolbachia* levels in a wide range of hosts including *Brugia malayi*, *D. mel*, and mosquito cell lines (Voronin *et al.*, 2012). *Wolbachia* infection was shown to induce autophagy in host cells, however, upon induction of autophagy, *Wolbachia* levels were reduced. Interestingly, it has also been shown that autophagy negatively regulates *wnt* signaling by promoting the degradation of Disheveled. It is possible that *wnt* signaling is modulating the effect of autophagy on *Wolbachia*, since our data indicate that a reduction in *wnt* signaling also reduces *Wolbachia* levels (*arm-RNAi*).

Other studies have shown that introduction of *Wolbachia* into previously uninfected mosquito cell lines up-regulates the 26S proteasome (Fallon *et al.*, 2009). The lack of metabolic pathways in the *Wolbachia* genome but retention of pathways for amino acid uptake and metabolism supports proteasomal activation as a mechanism to degrade host proteins and support *Wolbachia* growth. miRNAs have also been implicated in contributing to *Wolbachia* intracellular titers (Hussain *et al.*, 2011; Zhang *et al.*, 2013). It was found that *Wolbachia* infection upregulates *ae-miR2940*, which then facilitates *Wolbachia*

infection. miRNAs have also been shown to modulate Armadillo protein level, by stabilizing the protein and preventing proteasomal degradation (Pancratov *et al.*, 2013).

Understanding the mechanisms by which *Wolbachia* accumulate in certain cells and tissues at high densities can have major implications in the field. It has been shown that *Wolbachia* can reduce the vector competence of mosquitos to several different pathogens (Kambris *et al.*, 2009; Moreira *et al.*, 2009; Bian *et al.*, 2010; Walker *et al.*, 2011; Blagrove *et al.*, 2012; Bian *et al.*, 2013). One model supporting *Wolbachia*-mediated reductions in vector capacity is metabolic competition where the symbiont and the pathogen are competing for the same resources (Tortosa *et al.*, 2008; Moreira *et al.*, 2009). A correlation between *Wolbachia* densities and virus interference is one of the several lines of evidence supporting this model (Osborne *et al.*, 2009; Frentiu *et al.*, 2010; Lu *et al.*, 2012; Osborne *et al.*, 2012; Chrostek *et al.*, 2013). With the data presented here, if we could induce tropism to certain tissues with high pathogen loads, for example the gut or salivary glands, we could potentially reduce vector capacity even further. Other potential applications for this study could be to induce tropism to the germline of mosquitoes that cannot establish a stable vertical transmission of *Wolbachia*, such as *Anopheles*, which are naturally uninfected with *Wolbachia*. Further investigation into the Wnt pathway and identification of both host and bacterial mechanisms of tissue tropism is fundamental in understanding how *Wolbachia* infect their hosts in nature and can provide novel applications for vector and disease control.

Figure 5.1: *Wolbachia* accumulates in the hub throughout the course of development

A-F. Representative images of hubs infected with *wMel* (A, C, E) and *wMelCS* (B, D, F) at different developmental time points: L3 (A and B), Newly Eclosed Adults (NE, C and D), and 7 day old adults (7d, E and F). DNA in blue, *Wolbachia* in green, and hub marker in red. **G.** Quantification of the frequency of hub tropism. The frequency of targeting the hub does not significantly differ between L3, NE, and 7d for *wMel* or *wMelCS* (Chi-square: $P=0.167$ and $P=0.218$, respectively). **H.** Quantification of density of *Wolbachia* in the hub. *wMel* significantly increases in density throughout development (t-test, $*P<0.05$). *wMelCS* significantly increases in density from NE adult to 7 day old adult, but not from L3 to NE (t-test, $****P<0.0001$ and $P>0.05$, respectively).

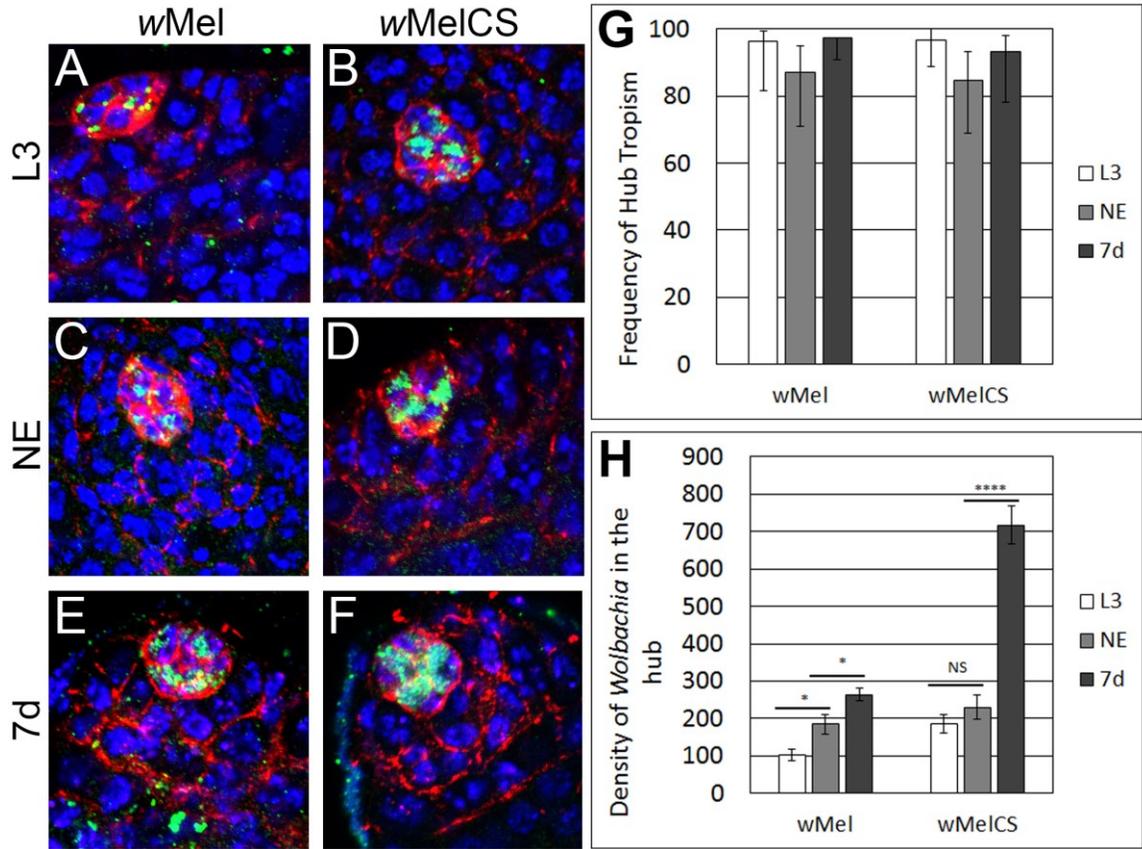


Figure 5.2 *Wolbachia* replicate more within the hub than in the surrounding tissue

A. Diagram and representative image of dispersed FtsZ (green) distribution in a non-dividing *Wolbachia* (red). **B.** Diagram and representative image of localized FtsZ (green) at the septation ring in a dividing *Wolbachia* (red). **C.** Representative image of regions quantified for *Wolbachia* division (Hub masked in red, Surrounding germline and soma masked in yellow). The total number of dividing and non-dividing *Wolbachia* were counted in each region. **D.** Representative image of a hub (white outline) with dividing *Wolbachia* (yellow arrowheads) and a clump of uncountable *Wolbachia* (red arrowhead). Green arrowhead points to a non-dividing *Wolbachia* outside the hub. **E.** Quantification of the relative fraction of dividing *Wolbachia* wMel in the hub and surrounding tissue. There is a significantly higher fraction of dividing *Wolbachia* in the hub (t-test, $P < 0.0001$).

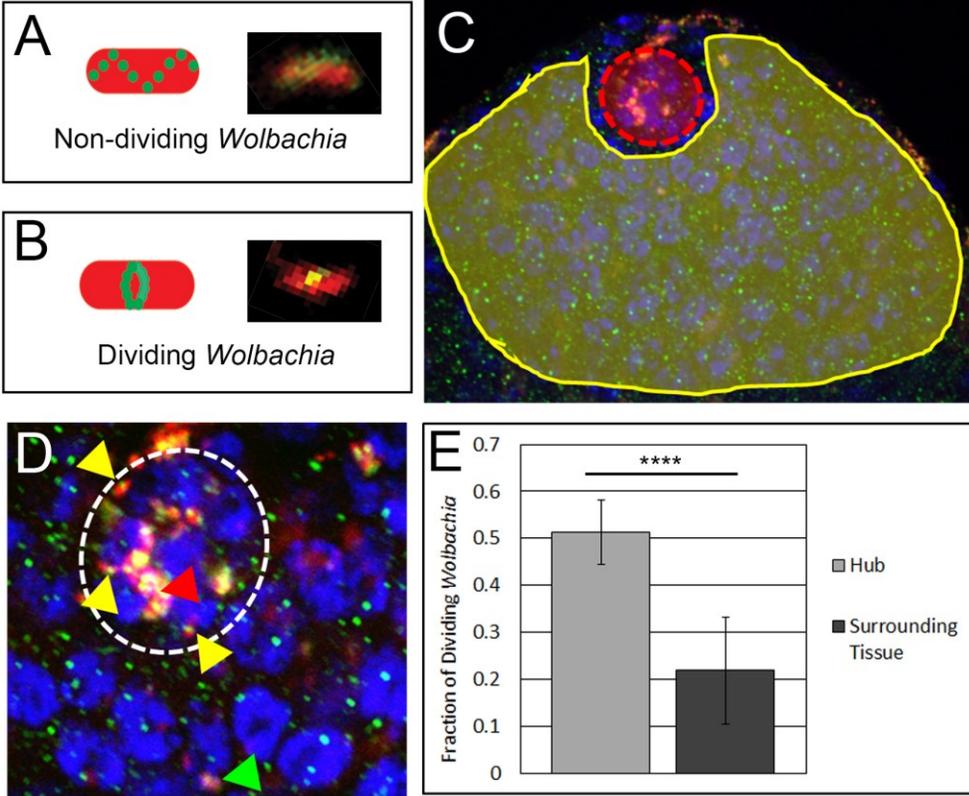


Figure 5.3 Tetracycline blocks *Wolbachia* wMel accumulation in the hub, but not initial targeting

A-E. Representative images of wMel *Wolbachia* densities in the hub under various treatment conditions. *Wolbachia* in green, DNA in blue, and hub marker in red. **A.** Hub of newly eclosed fly, with no tetracycline treatment (NE No Tet). **B.** Hub of newly eclosed fly, with tetracycline treatment (NE Tet). **C.** Hub of 7 day old fly, with no tetracycline treatment. **D.** Hub of 7 day old fly, raised on tetracycline until newly eclosed, then removed and aged 7 days without tetracycline. **E.** Hub of 7 day old fly continuously fed tetracycline throughout development and adult aging. **F.** Quantification of frequency of hub targeting under various tetracycline treatment conditions. Letters represent statistically significantly distinct groups. Error bars represent 95% confidence intervals. Flies not treated with tetracycline have *Wolbachia* tropism frequencies ~80%. If treated with tetracycline during larval development, frequency of tropism drops to ~50-60%, and does not recover with the removal of tetracycline during aging. **G.** Quantification of density of *Wolbachia* in the hub under various tetracycline treatment conditions. Letters represent statistically significantly distinct groups. Error bars represent SEM. *Wolbachia* densities significantly recover upon the removal of tetracycline during aging (7d Tet-> No Tet compared to 7d Tet->Tet treatment; t-test $P < 0.0001$).

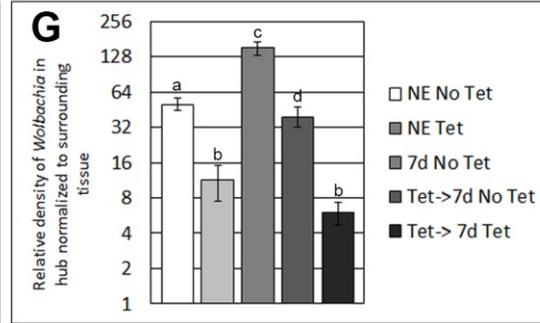
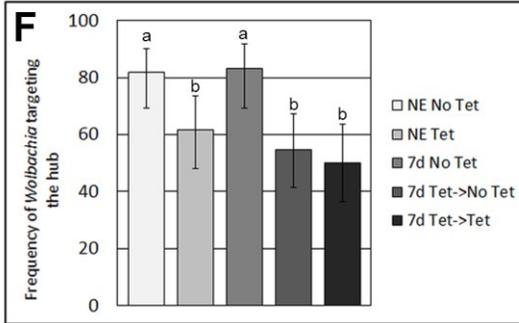
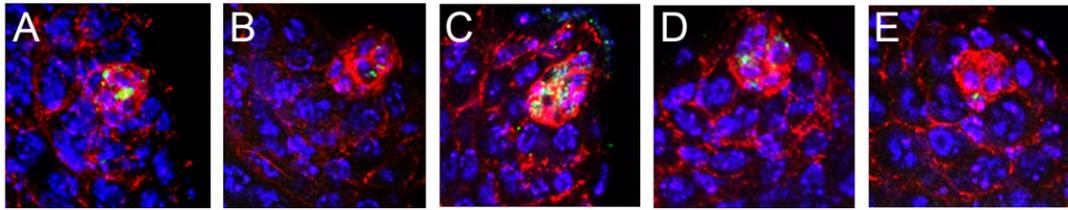


Figure 5.4: Tetracycline blocks *Wolbachia* wMelCS accumulation in the hub, but not initial targeting

A-E. Representative images of wMelCS *Wolbachia* densities in the hub under various treatment conditions. *Wolbachia* in green, DNA in blue, and hub marker in red. **A.** Hub of newly eclosed fly, with no tetracycline treatment (NE No Tet). **B.** Hub of newly eclosed fly, with tetracycline treatment (NE Tet). **C.** Hub of 7 day old fly, with no tetracycline treatment. **D.** Hub of 7 day old fly, raised on tetracycline until newly eclosed, then removed and aged 7 days without tetracycline. **E.** Hub of 7 day old fly continuously fed tetracycline throughout development and adult aging. **F.** Quantification of frequency of hub targeting under various tetracycline treatment conditions. Letters represent statistically significantly distinct groups. Error bars represent 95% confidence intervals. Flies not treated with tetracycline have *Wolbachia* tropism frequencies ~80%. If treated with tetracycline during larval development, frequency of tropism drops to ~50%, and does not recover with the removal of tetracycline during aging. **G.** Quantification of density of *Wolbachia* in the hub under various tetracycline treatment conditions. Letters represent statistically significantly distinct groups. Error bars represent SEM. *Wolbachia* densities significantly recover upon the removal of tetracycline during aging (Tet->7d No Tet compared to continuous Tet treatment; t-test $P < 0.0001$).

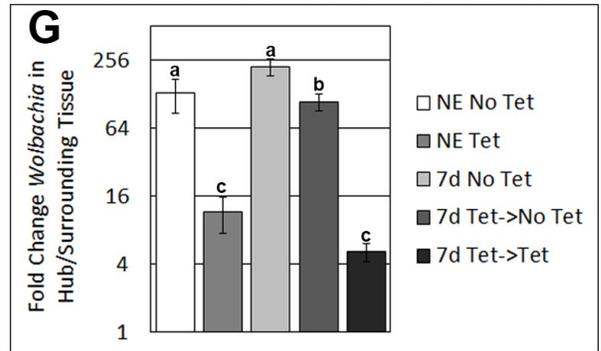
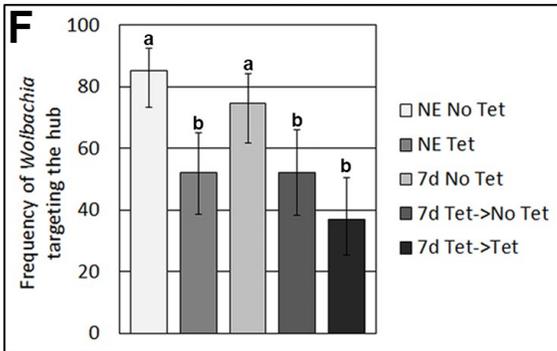
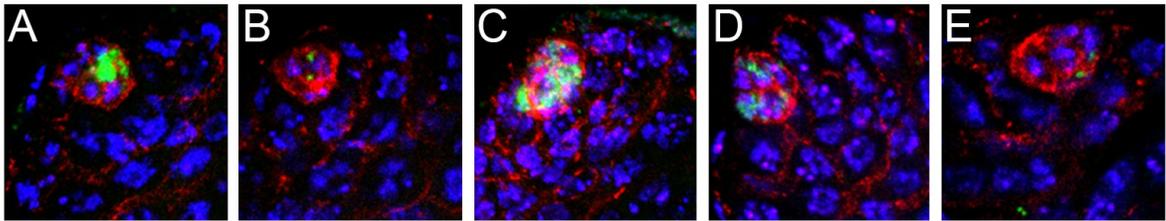


Figure 5.5: Unpaired levels do not affect *Wolbachia* titers in the hub

A and **B**. Quantification of *Wolbachia* tropism frequency (**A**) and density (**B**) in hubs expressing *Upd-RNAi* and sibling controls lacking the RNAi construct. There is no significant difference in frequency (test for differences in proportions $P > 0.05$ for both *wMel* and *wMelCS*) or density (t-test $P > 0.05$ for both *wMel* and *wMelCS*). **C** and **D**. Quantification of *Wolbachia* tropism frequency (**C**) and density (**D**) in hubs overexpressing *unpaired* and sibling controls lacking the overexpression construct. There is no significant difference in frequency (test for differences in proportions $P > 0.05$ for both *wMel* and *wMelCS*) or density (t-test $P > 0.05$ for both *wMel* and *wMelCS*).

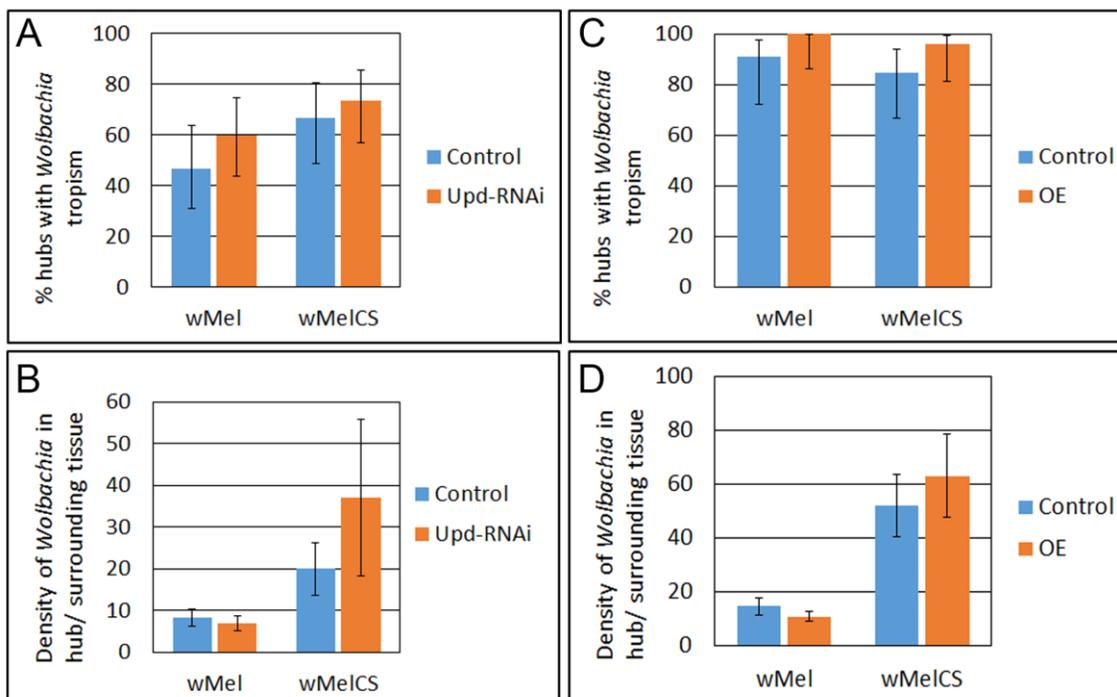


Figure 5.6: *unpaired* mRNA levels in *upd-RNAi* and *upd* overexpression testis

qPCR of *upd* mRNA levels in whole testis expressing RNAi or overexpression of *upd* in the hub (compared to control flies) indicated a modest reduction (A, 30-60%) and increase (B, 30-60%) in *upd* levels, respectively.

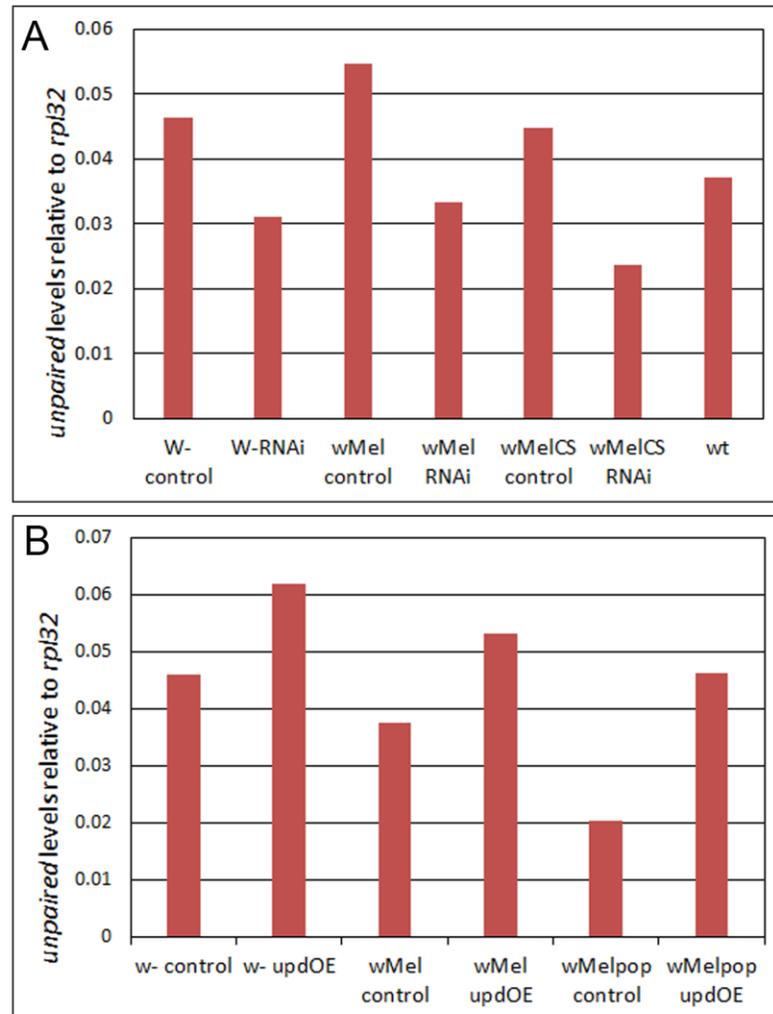


Figure 5.7 Assessment of RNAi-mediated knockdown of *armadillo*

Representative images of immunohistochemical assessment of *armadillo* knockdown. DNA is shown in blue, *Wolbachia* in green, Armadillo in white, and DN-Cadherin hub marker in red. **A.** Control fly lacking *UAS-armRNAi* construct shows robust Armadillo antibody binding to the hub cell surface, masking the DN-Cadherin antibody. **A'.** Gray scale image of hub labelled with DN-Cadherin. **A''.** Gray scale image of Armadillo channel showing robust labelling of the hub. **B.** *upd-GAL4>UAS-armRNAi* in the hub showing robust knockdown of Armadillo in the hub by lack of antibody staining. **B'.** Gray scale image of hub labelled with DN-Cadherin. **B''.** Gray scale image of Armadillo channel showing robust knockdown of Arm in the hub.

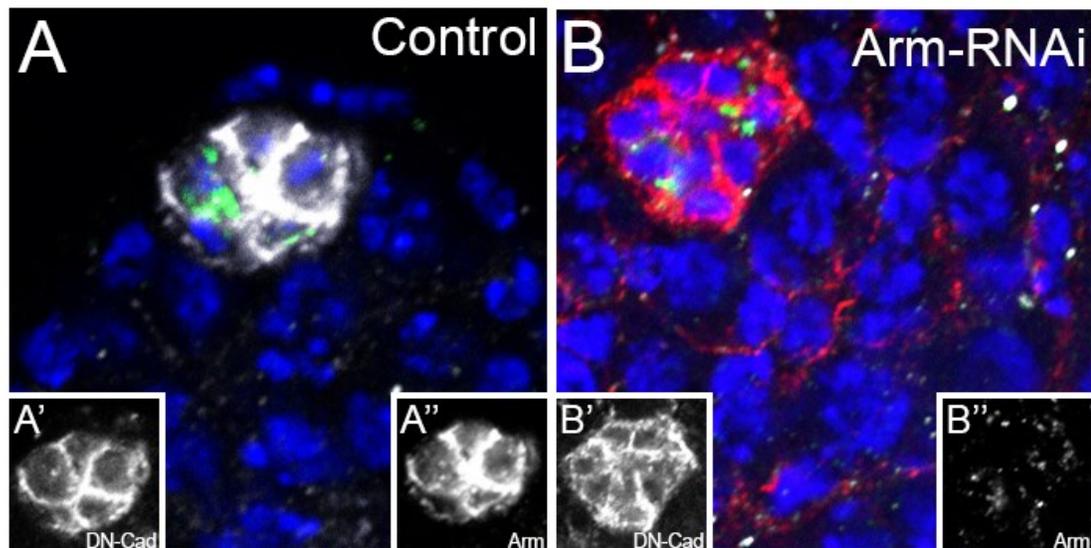


Figure 5.8 RNAi mediated knockdown of *armadillo* in the hub cells results in reduced *Wolbachia* densities.

A-D. Representative images of *Wolbachia*-infected hubs in control flies (*upd-GAL4*>; **A** and **C**) and RNAi flies (*upd-GAL4*>*Arm-RNAi*; **B** and **D**) infected with *wMel* (**A** and **B**) or *wMelCS* (**C** and **D**). Hub marker in red, *Wolbachia* in green, and DNA in blue; Inset of the hub showing *Wolbachia* channel in grayscale. **E.** Quantification of the average density of *Wolbachia* in the hub normalized to the surrounding germline and soma. *Wolbachia* density is significantly reduced in hubs expressing *arm-RNAi* (t-test: *wMel* $P < 0.0001$; *wMelCS* $P = 0.022$).

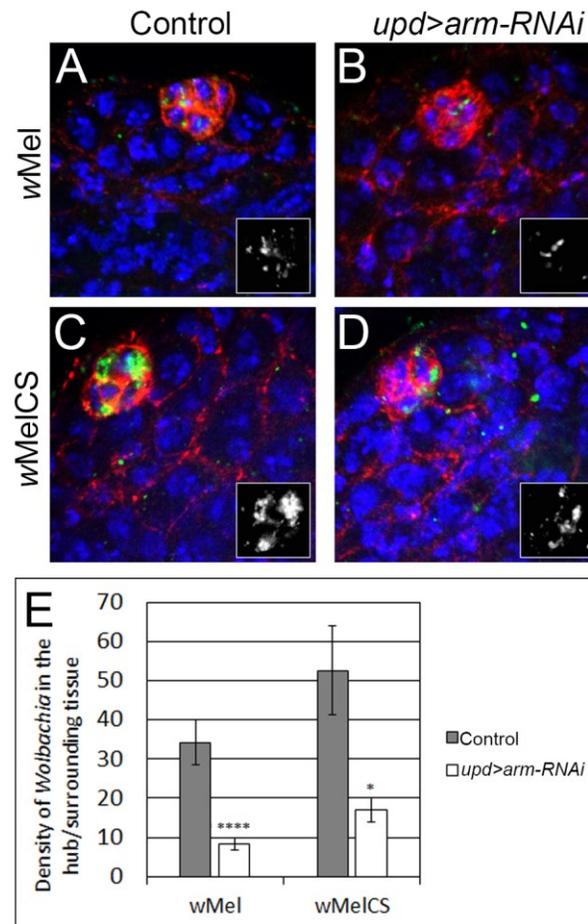


Figure 5.9 Assessment of RNAi-mediated knockdown of *DE-cadherin*

Representative images of immunohistochemical assessment of *DE-cadherin* knockdown. DNA is shown in blue, *Wolbachia* in green, DE-Cadherin in white, and DN-Cadherin hub marker in red. **A.** Control fly lacking *UAS-DEC-RNAi* construct shows robust DEC antibody binding to the hub cell surface, masking the DN-Cadherin antibody. **A'.** Gray scale image of hub labelled with DN-Cadherin. **A''.** Gray scale image of DEC channel showing robust labelling of the hub. **B.** *upd-GAL4>UAS-DEC-RNAi* in the hub showing robust knockdown of DEC in the hub by lack of antibody staining. **B'.** Gray scale image of hub labelled with DN-Cadherin. **B''.** Gray scale image of DEC channel showing robust knockdown of Arm in the hub.

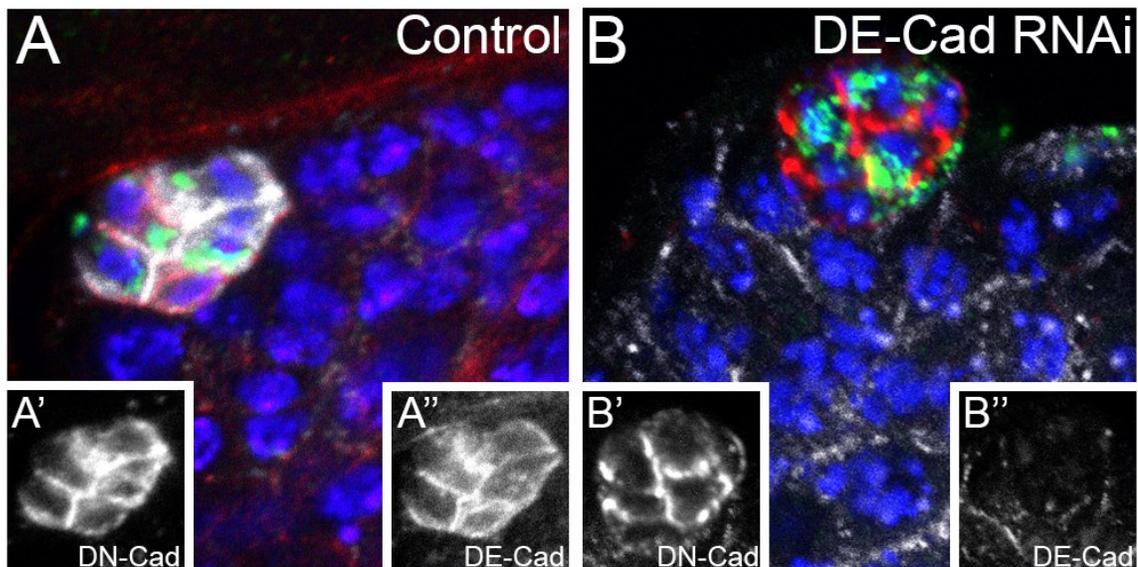


Figure 5.10: Armadillo does not modulate *Wolbachia* titers through adherens junctions

Quantification of the density of *Wolbachia* in the hub relative to the surrounding germline and soma in flies expressing *DEC-RNAi* and sibling control flies lacking the *RNAi* construct. *Wolbachia* is not significantly reduced in *DEC-RNAi* hubs.

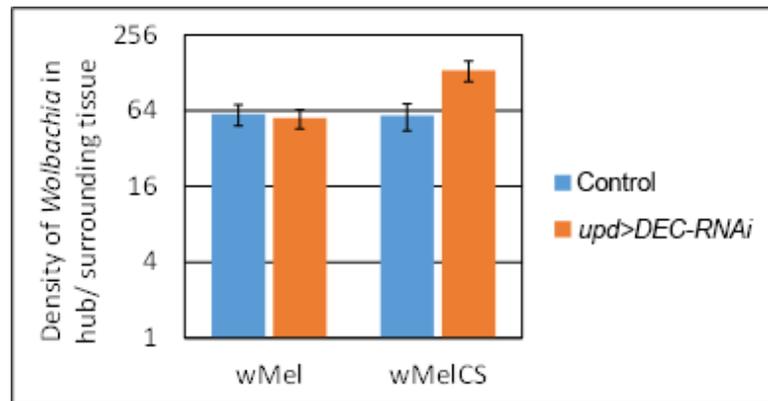


Figure 5.11: Expression of *armadillo*^{S10} in the hub cells results in increased *Wolbachia* densities.

A-D. Representative images of *Wolbachia*-infected hubs in control flies (*upd-GAL4*>; **A** and **C**) and OE flies (*upd-GAL4*>*arm*^{S10}; **B** and **D**) infected with *wMel* (**A** and **B**) or *wMelCS* (**C** and **D**). Hub marker in red, *Wolbachia* in green, and DNA in blue; Inset of the hub showing *Wolbachia* channel in grayscale. **E.** Quantification of the average density of *Wolbachia* in the hub normalized to the surrounding germline and soma. *Wolbachia* density is significantly increased in hubs expressing *armadillo*^{S10}. Error bars represent standard deviation. (**P<0.01, ***P<0.001)

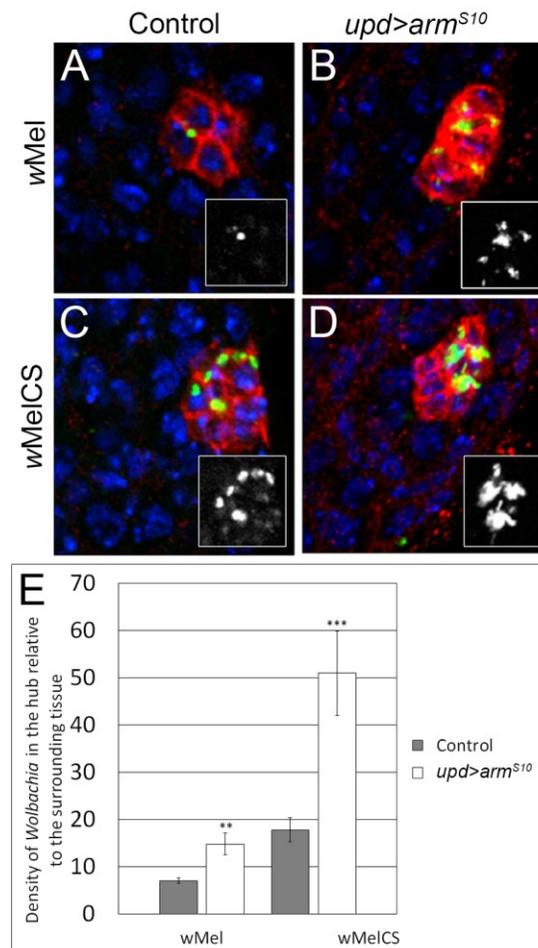


Figure 5.12: RNAi mediated knockdown of *armadillo* in the polar cells results in reduced *Wolbachia* densities

A-D. Representative images of *Wolbachia*-infected polar cells (PCs) in control flies (*upd-GAL4/+>*; **A** and **C**) and RNAi flies (*upd-GAL4/+>Arm-RNAi*; **B** and **D**) infected with *wMel* (**A** and **B**) or *wMelCS* (**C** and **D**). PC marker in red, *Wolbachia* in green, and DNA in blue; Inset of the PCs showing *Wolbachia* channel in grayscale. **E.** Quantification of the average density of *Wolbachia* in the PCs normalized to the surrounding follicle cells. *Wolbachia* density is significantly reduced in PCs expressing *arm-RNAi* (t-test: *wMel* P=0.0012; *wMelCS* P=0.0076).

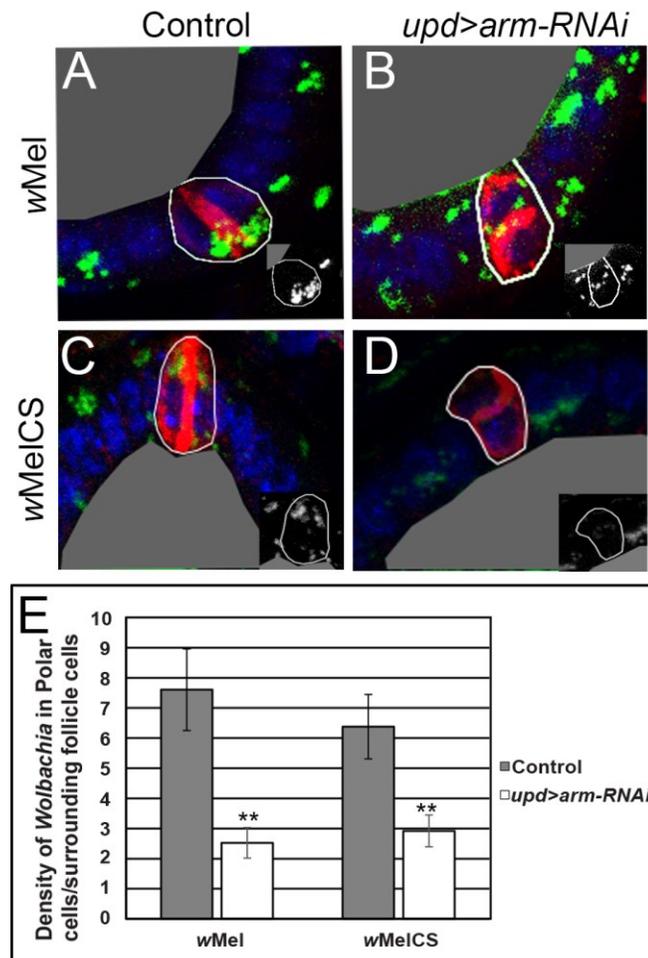


Table 5.1 Quantification of *Wolbachia* hub tropism throughout development

<i>Wolbachia</i> strain	Developmental Stage	N	HN	Frequency	Density
wMel	L3	27	26	96.30	102.41
	NE	31	27	87.10	184.66
	7d	39	38	97.44	264.23
wMelCS	L3	31	30	96.77	185.73
	NE	33	28	84.85	230.38
	7d	29	27	93.10	717.41

Hub tropism of wMel and wMelCS in L3 (third instar larvae), NE (newly eclosed adults), and 7d (7 day old adults). N indicates number of hubs analyzed. HN indicates the number of hubs with high niche (HN) infection. Average density of *Wolbachia* in the hub was measured using fluorescence intensity and was thresholded for background staining.

Table. 5.2 Quantification of *Wolbachia* replication in hub versus surrounding tissue

	# Testis	Total # <i>Wolbachia</i> counted	# Dividing
Hub	9	80	42
Surrounding	10	143	29

Wolbachia were stained using an *in situ* probe against the 16s rRNA of *Wolbachia*. The localization of FtsZ was assessed in discernably single *Wolbachia* cells. If FtsZ was distributed in the *Wolbachia* it was counted as non-dividing; if it was localized to the center of the *Wolbachia* it was considered dividing. Ambiguous FtsZ localization as well as clumped *Wolbachia* were not counted. The total number of discernably dividing or nondividing *Wolbachia* were counted in the hub and in the surrounding soma and germline.

Table 5.3 Quantification of *Wolbachia* hub tropism in tetracycline treated tissue

Sample	N	HN	Frequency	Density
<i>w</i> Mel				
NE No Tet	50	41	82.00	50.19
7d NoTet/NoTet	42	35	83.33	151.53
NE Tet	52	32	61.54	11.26
7d Tet/No Tet	53	29	54.72	39.38
7d Tet/Tet	48	24	50.00	6.00
<i>w</i> MelCS				
NE No Tet	54	46	85.19	130.26
7d NoTet/NoTet	55	41	74.55	223.59
NE Tet	50	26	52.00	11.66
7d Tet/No Tet	46	24	52.17	109.36
7d Tet/Tet	54	20	37.04	5.14

For description of the samples see Figure 2.3. N indicates number of hubs analyzed for frequency measurement. HN indicates number of niches highly infected with *Wolbachia*. Density measurements are based on the first 20 hubs in each sample.

Table 5.4 Quantification of *Wolbachia* hub tropism in *upd*-RNAi hubs

Sample	N	HN	Frequency	Density
wMel Control	32	15	46.88	8.32
wMel <i>upd-RNAi</i>	35	21	60.00	7.02
wMelCS Control	30	20	66.67	20.02
wMelCS <i>upd-RNAi</i>	34	25	73.53	36.97

Frequency was calculated based on the number of highly infected niches (HN) divided by the total number of hubs analyzed (N). Density was measured in the hub and normalized to the surrounding tissue.

Table 5.5 Quantification of *Wolbachia* hub tropism in *upd*-overexpressing hubs

Sample	N	HN	Frequency	Density
wMel Control	22	20	90.91	14.49
wMel <i>upd</i> -OE	24	24	100.00	10.74
wMelCS Control	26	22	84.62	51.80
wMelCS <i>upd</i> -OE	26	25	96.15	62.91

Frequency was calculated based on the number of highly infected niches (HN) divided by the total number of hubs analyzed (N). Density was measured in the hub and normalized to the surrounding tissue.

Table 5.6 Quantification of *Wolbachia* hub tropism in *arm*-RNAi hubs

Sample	N	HN	Frequency	Density
<i>w</i> Mel Control	26	18	69.23	34.22
<i>w</i> Mel <i>arm</i> -RNAi	24	18	75.00	8.40
<i>w</i> MelCS Control	26	16	61.54	52.62
<i>w</i> MelCS <i>arm</i> -RNAi	22	10	45.45	17.02

Frequency was calculated based on the number of highly infected niches (HN) divided by the total number of hubs analyzed (N). Density was measured in the hub, normalized to the surrounding tissue, and averaged across all HN samples. Efficiency of the knockdown was assessed based on antibody staining against Arm.

Table 5.7 Quantification of *Wolbachia* hub tropism in *DEC*-RNAi hubs

Sample	N	HN	Frequency	Density
wMel Control	32	32	100.00	59.93
wMel <i>upd>DEC-RNAi</i>	32	30	93.75	55.91
wMelCS Control	30	23	76.67	58.58
wMelCS <i>upd>DEC-RNAi</i>	32	26	81.25	133.83

Frequency was calculated based on the number of highly infected niches (HN) divided by the total number of hubs analyzed (N). Density was measured in the hub, normalized to the surrounding tissue, and averaged across all HN samples. Efficiency of the knockdown was assessed based on antibody staining against DEC.

Table 5.8 Quantification of *Wolbachia* hub tropism in *arm^{S10}*-overexpressing hubs

Sample	N	HN	Frequency	Density
wMel Control	46	21	45.65	7.01
wMel <i>upd>arm^{S10}</i>	52	19	36.54	14.78
wMelCS Control	34	21	61.76	17.78
wMelCS <i>upd>arm^{S10}</i>	36	19	52.78	50.95

Frequency was calculated based on the number of highly infected niches (HN) divided by the total number of hubs analyzed (N). Density was measured in the hub and normalized to the surrounding tissue and averaged across all HN samples.

CHAPTER 6

Summary and Future Perspectives

6.1 Summary of findings

Wolbachia are obligatory intracellular bacteria that infect up to 70% of all insect species, which can alter host reproduction in diverse ways. *Wolbachia* have emerged as a novel paradigm to control vectors that spread devastating infectious diseases such as malaria and Dengue virus. It has been shown that *Wolbachia* infection can prevent viral replication and dissemination within a host through upregulation of immunity pathways and metabolic competition. Even though *Wolbachia* is becoming an important tool for controlling disease vectors, little is known about the cellular and molecular events occurring at the host-pathogen interface.

Tissue tropism, the tissues preferentially colonized by the bacteria, is a fundamental aspect of host-microbe interactions, which ultimately dictates sites of infection and microbial transmission. *Wolbachia* has a strong tropism for the female germline, where they are vertically transmitted to the next generation, through the egg cytoplasm. Although the primary mode of transmission of *Wolbachia* is vertical, there is also evidence of extensive horizontal transmission, as the phylogenies of the hosts and bacteria are not congruent. The mechanisms underlying both vertical and horizontal transmission are not well understood.

In Chapter 3, we investigated the cellular mechanisms of vertical transmission. In a survey of 11 different *Drosophila-Wolbachia* pairs, we found that tropism to the SSCN is conserved. We hypothesize that the conservation of tropism to the SSCN is due to a

selective pressure to colonize the germline and ensure vertical transmission. In addition, we found a secondary tropism to the GSCN in some species. Through introgression and transinfection experiments, we show that the differential targeting of these two stem cell niches is dictated by the *Wolbachia* strain, rather than the *Drosophila* host species. Finally, we showed through extensive image analysis that germline cysts in proximity to highly infected niches harbored a higher density of *Wolbachia*, suggesting that stem cell niche tropism is facilitating vertical transmission through passage of *Wolbachia* to the germline. Stem cell niche tropism in the ovary has been discovered in other, less well characterized hosts including the filarial worm (Landmann *et al.*, 2012), bed bug (Hosokawa *et al.*, 2010), and the leaf hopper (Sacchi *et al.*, 2010), allowing for the future investigation of stem cell niche tropism as a generalized mechanism for *Wolbachia* vertical transmission.

In Chapter 4 we investigated the conservation of niche tropism to the stem cell niche in the males, the hub. If the major role of niche tropism is related to *Wolbachia* transmission, evolutionary theory predicts that there should be reduced selective pressure to maintain niche tropism in males, since *Wolbachia* is not transmitted through the sperm. Indeed, we found a lack of conservation of hub tropism in *Drosophila* males, where only 6 out of the 9 species investigated displayed *Wolbachia* hub tropism. Furthermore, we found fundamental differences in the underlying mechanisms driving niche tropism in the males, as compared to the females. Introgression experiments suggested that both the host and the *Wolbachia* strain contribute to the mechanisms of niche tropism in the males. However, when introgressed into the same genetic background, *Wolbachia* is still capable of driving differential tropism to the hub, as evidenced by the several different *wMel* strains

of *Wolbachia* introgressed into the same isogenized host genetic background. These experiments further suggested that hub tropism in *Drosophila* is a rapidly diverging phenotype, as these *Wolbachia* strains diverged from a single ancestor only 8,000 years ago.

Chapter 5 investigated the cellular and molecular mechanisms involved in hub tropism in *D. melanogaster*. We found, through a developmental profile of hub tropism, that *Wolbachia* target the hub early in development, and accumulate to high densities as the fly develops and ages. Several lines of evidence support a model of preferential replication of *Wolbachia* in the niche as compared to the surrounding soma and germline, including the fraction of *Wolbachia* dividing in the hub relative to the surrounding soma and germline. Towards identifying a host derived molecular mechanism for hub tropism, we conducted a targeted screen to knock down host proteins involved in stem cell and hub biology. We found a role for Armadillo (Arm, *Drosophila* β -catenin) in modulating *Wolbachia* density in the hub. Upon RNAi-mediated knockdown of Arm, we observed a reduction of *Wolbachia* levels. Overexpression of a constitutively active Arm (*arm*^{S10}) confirms Arm's role in contributing to *Wolbachia* titers in the hub, as an increase in *Wolbachia* densities were observed. The overexpression experiments also implicate Wnt signaling as the overall mechanism, as the *arm*^{S10} construct drives an increase in the transcription of Wnt responsive genes. More experiments are necessary, however, to confirm the role of Wnt signaling in *Wolbachia* hub tropism.

6.2 Significance and future directions

These results have identified novel mechanisms for bacterial tissue tropism as well as for vertical transmission of *Wolbachia*. Given the widespread occurrence of stem cell niche tropism across the *Drosophila* genus, as well as in other insect species, these findings could have wide implications for investigations of *Wolbachia* transmission in nature and in the use of *Wolbachia* as a control agent for the spread of infectious diseases transmitted by insect vectors. More broadly, bacteria exploiting properties of host stem cell biology is a previously overlooked aspect of pathogenesis. There are a few recent examples of other bacteria which take advantage of stem cell biology to promote infection (summarized in Table 6.1). *Helicobacter pylori* is generally an extracellular pathogen, however, intracellular bacterial collections are found in gastric epithelial progenitors, which act as a repository for *H. pylori* (Oh *et al.*, 2005). *Mycobacterium leprae* is known to infect the Schwann cells in the adult nervous system and reprogram them into progenitor like stem cells, directing differentiation of these cells to promote the dissemination of infection (Masaki *et al.*, 2013). Understanding the mechanisms of how *Wolbachia* target the stem cell niches in *Drosophila* could also provide insight into human disease-related stem cell-microbe interactions.

6.2.1 Identification of *Wolbachia* factors driving stem cell niche tropism

Chapters 3 and 4 established the foundation for using a comparative genomics approach to identify *Wolbachia* related factors driving stem cell niche tropism. In Chapter 3, we surveyed 11 different *Wolbachia* strains for tropism in the *Drosophila* ovary. Several of these *Wolbachia* strains have sequenced genomes and can be used for comparative

genomics. A disadvantage to this approach is the highly divergent, recombining *Wolbachia* genome (Klasson *et al.*, 2009; Siozios *et al.*, 2013). Given this, it would be difficult to attribute *Wolbachia* tropism patterns in the ovary to a single gene. Chapter 4 continued the survey of niche tropism in the male gonads. In addition to the survey of different *Drosophila* species, this analysis examined differences in hub tropism of very closely related *Wolbachia* strains. The several wMel *Wolbachia* strains analyzed in this study have been sequenced and their genomes are fully assembled (Chrostek *et al.*, 2013). This analysis offers the opportunity of comparing niche tropism phenotypes in *Wolbachia* strains with fewer genomic differences, allowing for a more focused genomic study on *Wolbachia* factors driving differences in niche tropism.

6.2.2 Further characterization of host factors driving stem cell niche tropism

There is some insight into the intracellular factors which promote *Wolbachia* accumulation, including autophagy (Voronin *et al.*, 2012), proteasomal degradation (Fallon *et al.*, 2009), microRNAs (Hussain *et al.*, 2011; Zhang *et al.*, 2013), and host cytoskeleton (Ferree *et al.*, 2005). Experiments detailed in Chapter 5 revealed a mechanism in which Armadillo levels contribute to *Wolbachia* levels in the hub and polar cells, potentially through Wnt signaling. To further confirm Wnt signaling as the pathway through which Armadillo affects *Wolbachia* levels, it will be important to analyze the effect of other genes in the Wnt pathway on *Wolbachia* levels. The single protein downstream of Arm in the Wnt pathway is dTCF/pangolin. Pangolin is the protein binding partner of Arm which binds to DNA to drive transcription of Wnt target genes. Several attempts have been made to target *pangolin* for RNAi-mediated degradation, as well as express a dominant-

negative form of *pangolin* in the hub to reproduce results found with *arm^{RNAi}*. These experiments are technically limited in the hub. The lack of an antibody for pangolin does not allow for the evaluation of the knockdown in RNAi experiments, and without this parameter, results of this experiment were inconclusive. Other constructs, such as those expressing a dominant-negative form of Pangolin, were lethal when expressed with the hub-specific driver. These experiments could be more easily performed in the polar cells of the ovary. The polar cells are specified in the adult fly, during oogenesis, and there is constant turnover of this cell type as new egg chambers are formed. The polar cell system also allows for easier clonal analysis using the Flp/FRT system. This approach could be used to investigate mutant alleles of Armadillo which are deficient in only Wnt signaling or only adherens junctions. This would allow for the distinction between reducing/increasing Armadillo levels directly effecting *Wolbachia* levels and the involvement of the Wnt pathway or adherens junctions.

If Wnt signaling is indeed contributing to *Wolbachia* tropism in the hub, it would also be interesting to identify the downstream Wnt targets responsible. There is vast literature investigating Wnt signaling in diverse hosts/tissues, however, there is limited knowledge of the downstream Wnt targets in the gonads. To address this question, it would be important to identify the target genes responsive to Wnt signaling in the hub. This could be achieved through transcriptional profiling of hubs expressing Armadillo-RNAi as compared to controls. Each differentially expressed gene could then be targeted for RNAi mediated knockdown in the hub to assess its effect on *Wolbachia* levels. Candidate genes

to focus on could be involved in any of the previously implicated pathways shown to affect *Wolbachia* levels, including autophagy, proteolysis, cytoskeleton, or miRNA pathway.

A bigger picture question stemming from these findings is: can tropism be induced by overexpressing Armadillo, or increasing Wnt signaling? This could be addressed on two levels, first, in a similar system/organism, and second, in a novel host. The feasibility of this hypothesis could be tested very simply in *D. mel* ovaries. *Wolbachia* wMel only target the SSCN in the ovary at high densities. There are GAL4 drivers that drive expression in the GSCN which could be used to express constructs which promote *Wolbachia* densities, such as *arm*^{S10}. However, in order for these experiments to be successful, the cells need a founder *Wolbachia* population to expand. If we can drive tropism of wMel to the GSCN, the applications could be expanded to more diverse hosts. For example, *Anopheles* mosquitoes cannot successfully establish a vertically transmitted *Wolbachia* infection. Recent work has implicated the native microbiome as a factor inhibiting *Wolbachia* transmission in these mosquitoes (Hughes *et al.*, 2014). If we could drive *Wolbachia* tropism to the germline, it may be possible to establish a stable line of *Wolbachia* infected *Anopheles* mosquitoes, however it would also be important to address any adverse effects of increased *wnt* signaling.

6.3 Conclusion

The cellular and molecular interactions between *Wolbachia* and their hosts are not well understood. This thesis has shed light on the underlying aspects of *Wolbachia* transmission via stem cell niche tropism as well as the cellular and molecular mechanisms promoting stem cell niche tropism. A deeper understanding of the relationship between

Wolbachia and their host is important in several aspects. *Wolbachia*-based technologies are emerging as a promising tool for the control of vectors of deadly human diseases, including Dengue fever, West Nile Virus and malaria (Moreira *et al.*, 2009; Kambris *et al.*, 2010; Hoffmann *et al.*, 2011; Hughes *et al.*, 2011; Pan *et al.*, 2012). Introduction of *Wolbachia* into these disease vectors has been shown to reduce vector capacity and disease transmission. Understanding the basis of *Wolbachia* targeting of specific tissues in the host and its consequences towards bacterial transmission will provide further mechanistic insight into their extremely successful propagation and is also relevant for developing new *Wolbachia*-based vector control approaches.

Table 6.1: Examples of microbes which subvert stem cell biology to promote infection of host tissues and transmission

Microbe	Tropism	Relevance to pathogenesis	Reference
<i>Helicobacter pylori</i>	GEPs	generally extracellular, but infects GEPs to function as a repository	(Oh <i>et al.</i> , 2005)
<i>Mycobacterium leprae</i>	Schwann cells	reprograms Schwann cells to progenitor stem-like cell to direct differentiation, resulting in bacterial dissemination	(Masaki <i>et al.</i> , 2013)
<i>Mycobacterium tuberculosis</i>	BM-MSCs	dormant Tb resides in BM-MSCs to evade immune system	(Das <i>et al.</i> , 2013)
<i>Wolbachia</i>	gonad stem cell niches	accumulates in the stem cell niches in the ovary to promote transmission	(Toomey <i>et al.</i> , 2013)

Table summarizing known bacteria which subvert host stem cell biology to promote infection. GEP, gut epithelial progenitors; BM-MSCs, bone marrow-mesenchymal stromal cells.

Appendix 1

Stem cell division in the *Drosophila mauritiana* testis

Portions of this appendix were previously published in (Fast *et al.*, 2011).

Wolbachia has been shown to modulate the division of the germline stem cell in the *D. mau* ovary in a niche dependent mechanism (Fast *et al.*, 2011). To assess if this finding was conserved in the testes, stem cell division was measured in *Wolbachia* infected (W+) and *Wolbachia* uninfected (W-) males. In W+ *D. mau* males, 65% of the hubs are highly infected with *Wolbachia* (Figure 4.1 and Table 4.1). In these niches (HN), the ratio of dividing stem cells per testis is 43% (Figure A1.1, Table A1.1). In contrast, in niches with low *Wolbachia* levels (LN) the ratio of stem cell division per testis is 29%, similar to that of non-infected testes (27%, Figure A1.1, Table A1.1). These results agree with findings in the females: the mechanism of *Wolbachia*-driven increase of stem cell mitosis is dependent on high density of the bacteria in the niche.

Figure A1.1: Average stem cell division in *Drosophila mauritiana* testis

In highly infected hubs (HN), there is an average of 0.43 dividing stem cells. In lowly infected hubs (LN), there is an average of 0.29 dividing stem cells, similar to that of W-hubs (0.27). Although these results are not statistically significant, they agree with trends found in the ovary, where GSCs adjacent to highly infected niches have a significantly higher rate of GSC division.

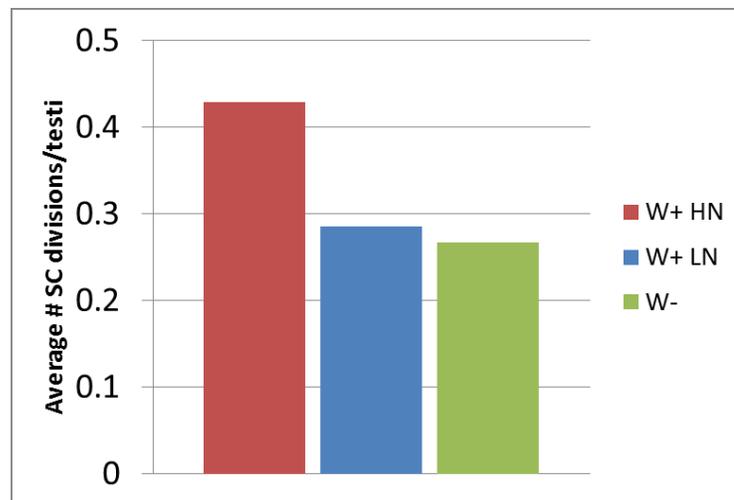


Table A1.1 Quantification of stem cell division in the *Drosophila mauritiana* testes

	N	# Dividing stem cells	Average
W+, HN	49	21	0.43
W+, LN	28	8	0.29
W-	30	8	0.27

Appendix 2

Wolbachia effect on stem cell biology

A2.1 Effects of *Wolbachia* hub tropism on *Drosophila melanogaster* stem cell biology

Previous work in our lab demonstrated that *Wolbachia* infection of the GSCN in *D. mau* modulates the rate of division of the adjacent stem cell (Fast *et al.*, 2011). This prompted us to investigate whether the hub tropism of *wMel* and *wMelCS* could modulate the division of the adjacent GSCs and CySCs. Using an antibody against phospho-histone H3 (labels cells in mitosis) and Vasa (germline marker), we measured the number of dividing GSCs and CySCs in W-, *wMel* infected, and *wMelCS* infected testes (Figure 5.2, Table 5.2).

When flies were aged to 7 days at 25°C, *Wolbachia* did not significantly alter the number of stem cells dividing. There was a typical 2 CySC: 1 GSC ratio of dividing cells in the three samples analyzed (Figure A2.1, Table A2.1) (de Cuevas *et al.*, 2011; Matunis *et al.*, 2012). However, when the flies were raised and aged to 7 days at 29°C, there was an effect. Irrespective of *Wolbachia* infection status, temperature alone affects GSC division, altering the ratio to 1 CySC: 1 GSC division. *Wolbachia* further induced significant variability in GSC division at 29°C, whereas the variability in CySC division was not significantly altered. When comparing the standard deviations between W+ and W- samples, for GSC division, both *wMel* and *wMelCS* have significantly larger standard deviations than W- (Figure A2.1B; F-test of equal variance: $P_{\text{mel}}=0.04$ and $P_{\text{cs}}=0.008$). For

CySC division, W- and *wMel* do not have significantly different variances ($P=0.267$) whereas *wMelCS* has a significantly lower variance than W- ($P=0.03$).

There are two possible hypotheses for the increase in variability in GSC division in *Wolbachia* infected testis: 1) Increase in GSC number, or 2) Increase in GSC division rate. To address this question, the number of GSCs and CySCs were counted in W-, *wMel*, and *wMelCS* infected flies (Figure A2.1C, Table A2.2). There was no significant difference in stem cell numbers between W+ and W- testis, for either GSCs or CySCs (Figure A2.1D, t-tests: W-/wMel GSCs $p=0.924$, W-/wMelCS GSCs $p=0.579$, W-/wMel CySCs $p=0.108$, W-/wMelCS CySCs $p=0.436$, Table A2.2). This suggests that *Wolbachia* may be inducing variability in GSC division rates. These data indicate that *Wolbachia* tropism to the hub does not have any effect on stem cell division when the flies are raised at 25°C. However, when raised at a slightly higher temperature, *Wolbachia* induce great variability in germline stem cell activity.

A2.2 Effects of *Wolbachia* hub tropism on hub cell number

In the previous analysis, the number of hub cells in W+ versus W- were also counted. Interestingly, there was a *Wolbachia* dependent significant difference in hub cell number. *wMel* and *wMelCS* infected flies both have 1.5-fold higher number of hub cells than W- (Figure A2.2, Table A2.3: Average number of hub cells: *wMelCS* = 7.4, *wMel* = 7.25, and W- = 4.79; $P<0.0001$). It is possible that *Wolbachia* could be causing more SGPs to become fated as hub cells, however more experiments are necessary to confirm this phenomenon and determine how *Wolbachia* are affecting this phenotype.

These data highlight the complexity of host microbe interactions. We identified a phenotype produced by an environmental change (germline stem cell division increases at 29°C) that has increased variability with *Wolbachia* infection. Stem cell/niche biology in the testes is very complex. There are multiple pathways, which intersect in various ways. An added layer of complexity is that the CySCs are considered part of the niche for the GSCs, as pathways that are activated in the CySCs by ligands secreted from the hub, promote GSC maintenance. Given all of these complexities, *Wolbachia* could be affecting one gene with multiple roles, or several genes with overlapping roles.

Figure A2.1: *Wolbachia* effect on stem cell division in the *Drosophila* testis

A. Percentage of hubs infected with *wMelCS*, *wMel*, and uninfected (W-) with dividing stem cells at 25°C. There is an average ratio of two CySCs dividing to every one GSC dividing. **B.** Percent hubs infected with *wMelCS*, *wMel*, and uninfected (W-) with dividing stem cells at 29°C. *Wolbachia* significantly increases the variability in GSC division, but not CySC division (F-test of equal variance: GSC division: W-/wMel P=0.04, W-/wMelCS P=0.008; CySC division: W-/wMel P=0.243; W-/wMelCS P=0.03 [W- variance significantly larger]). **C.** Representative image of counting stem cells. Hub marker in green (green arrowhead), Germline in white (GSC white arrowhead), CySCs in red (red arrowhead), and DNA in blue. Germline cells directly adjacent to the hub are GSCs. **D.** Quantification of stem cells in *wMelCS*, *wMel*, and uninfected testis. There is no significant difference in GSC or CySC number across samples (t-test P>0.05).

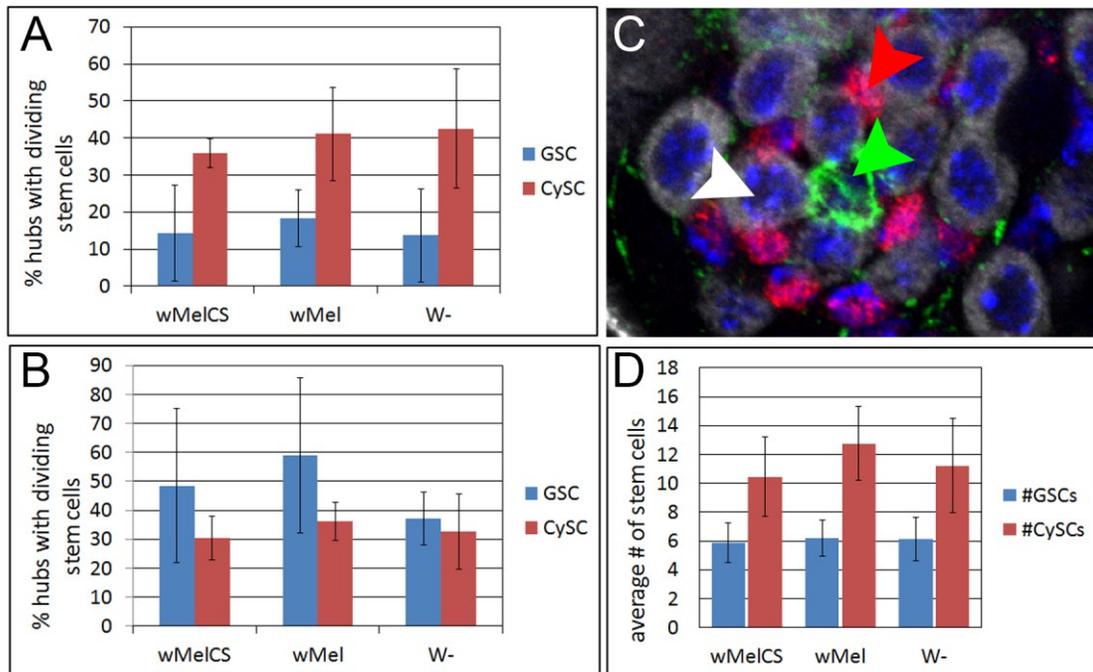


Figure A2.2: *Wolbachia* increases hub cell number in the *D. mel* testes

Hub cells were counted using DNA and a hub membrane marker to outline the hub cells.

Wolbachia infected testes have on average 2 more hub cells than *Wolbachia* uninfected hubs (pair-wise t-tests, $P < 0.0001$; t-test between *wMelCS* and *wMel*, $P = 0.76$).

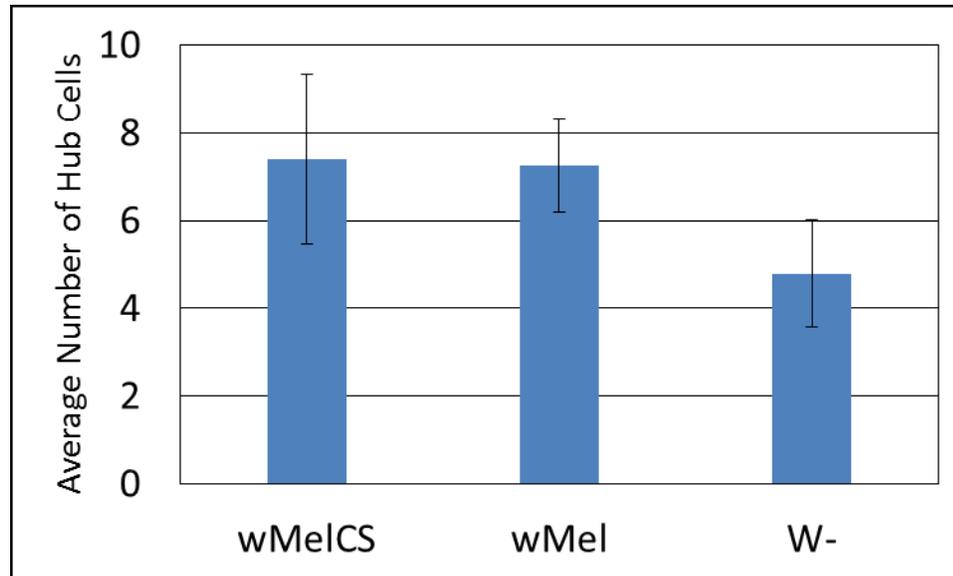


Table A2.1: Quantification of stem cell division in *Wolbachia* infected and uninfected testis

<i>Wolbachia</i>	Temperature	GSC Division	CySC Division
wMelCS	25°C	23.37	34.77
wMel	25°C	20.37	38.53
W-	25°C	21.65	41.08
wMelCS	29°C	48.54	30.40
wMel	29°C	58.93	36.20
W-	29°C	37.12	32.57

Dividing stem cells were quantified based using an antibody against phosphorylated histone-H3. Germline and cyst stem cells were identified based on their localization at the hub and staining with the germline marker, Vasa. Total number of dividing stem cells was normalized by the number of hubs analyzed.

Table A2.2: Quantification of stem cell number in *Wolbachia* infected and uninfected testis

<i>Wolbachia</i> strain	Average # GSC	Average # CySC
wMelCS	5.90	10.45
wMel	6.20	12.75
W-	6.16	11.21

The following antibodies were utilized in the identification and counting of stem cells: Vasa (germline), DE-Cadherin (hub), Zfh-1 (CySCs and daughter cells), and DNA was counterstained with Hoechst. GSCs (germline stem cells) were counted based on their localization at the hub and staining with Vasa. CySCs (cyst stem cells) were counted based on their localization at the hub and staining with Zfh-1.

Table A2.3: Quantification of hub cell number

<i>Wolbachia</i> strain	N	Average # of Hub Cells
wMelCS	20	7.40
wMel	20	7.25
W-	19	4.79

Appendix 3

Establishing methods for *Culex pipiens* investigations

A3.1 Antibody screening

To begin investigating mosquito oogenesis, we conducted a screen of antibodies which label proteins/structures in the *Drosophila* ovary to assess if they work in mosquito ovaries. The same immunohistochemistry protocol that is used for *Drosophila* was employed here (see Ch. 2.4.1). A summary of antibodies tested, the structures they label in *Drosophila*, and whether they worked in the mosquito is listed in Table A3.1.

A3.2 *In situ* hybridization of whole mosquitoes

To look at *Wolbachia* infection dynamics of whole mosquitoes, we developed a protocol for Fluorescent *in situ* hybridization of whole mosquitoes. The protocol is detailed in Ch. 2.4.2.2, but generally involves incubating the mosquitoes in Carnoy's solution for several days to reduce autofluorescence of the cuticle and then subjecting the mosquito to the ISH protocol. Also, the legs were removed and holes were poked into the abdomen for increased penetration of the *in situ* probe. Figure A3.1 displays a representative image of a whole mosquito's ovary with a probe against *Wolbachia* and counterstained with Hoechst.

Figure A3.1 : Representative images of *Wolbachia in situ* hybridization on whole mosquitoes

A. 10x magnification of mosquito abdomen, with *Wolbachia* (red) infected ovary. **B.** 60x magnification of a single germaria within the mosquito abdomen infected with *Wolbachia* (red), with DNA counterstained in blue.

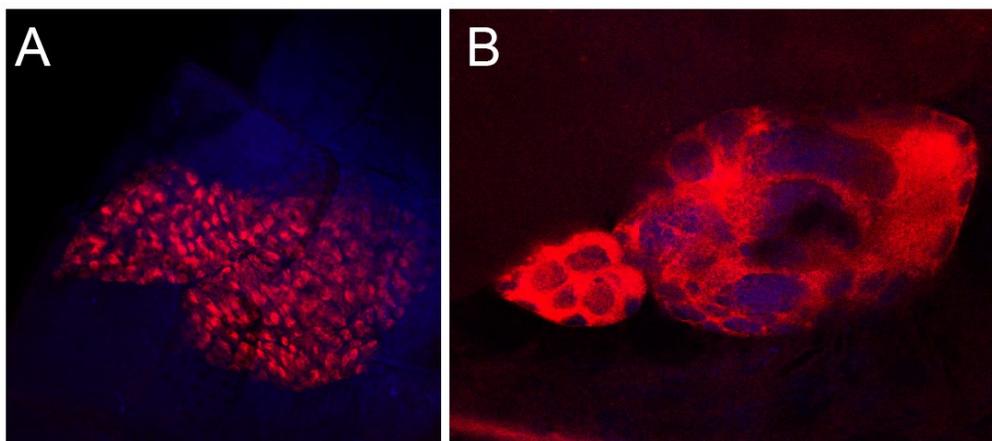


Table A3.1: Summary of antibodies tested in mosquito ovaries

Protein	Structure Labeled in <i>Drosophila</i>	Work?	Structure Labeled in Mosquito
Adducin	Fusome	N	
Adducin related proteins, Hts	Fusome	Y	Oocyte
α -catenin	Cap cells, Follicle cells	N	
α -spectrin	Spectrosome	N	
Armadillo	Cap cells, Follicle cells	N	
Fasciclin III	Polar cells	N	
Oskar	Germline	Y	Germline

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Zug, R. and P. Hammerstein (2012). "Still a host of hosts for Wolbachia: analysis of recent data suggests that 40% of terrestrial arthropod species are infected." PLoS One **7**(6): e38544.

CURRICULUM VITAE

MICHELLE E. TOOMEY OLSEN

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EDUCATION

PhD, Molecular and Cell Biology and Biochemistry January 2011- Present; Expected October 2014

Boston University, Boston, MA

Master of Arts, Molecular and Cell Biology and Biochemistry (GPA: 3.72) Sept 2008- Dec 2010

Boston University, Boston, MA

Transferred into PhD Molecular and Cellular Biology and Biochemistry Program

Bachelor of Science, Chemistry (GPA: 3.84) May 2008

Bridgewater State College, Bridgewater, MA

Major: Chemistry; Concentration: Biochemistry

HONORS AND AWARDS

2nd Place, Best Oral Presentation, 8th International *Wolbachia* Conference, Innsbruck Austria, June 2014

Terner Award for outstanding contribution to Molecular and Cell Biology and Biochemistry at Boston University (Summer stipend support), May 2013

1st Place, Best Talk, Biology Department Graduate Student Symposium, Boston University, May 2013

1st Place, Best Oral Presentation, 7th International *Wolbachia* Conference, Oleron France, June 2012

2nd Place, Best Talk, Biology Department Graduate Student Symposium, Boston University, May 2012

1st Place, Best Poster Award, 6th International *Wolbachia* Conference, Asilomar CA, June 2010

Summa cum laude, Bridgewater State College, 2008

RESEARCH EXPERIENCE

Boston University: Frydman Lab, May 2009-Present

Advisor: Dr. Horacio Frydman

Dissertation: *Cellular, molecular, and evolutionary mechanisms of Wolbachia stem cell niche tropism in Drosophila*

PUBLICATIONS

Toomey, ME and Frydman, HM. (2014) "Extreme divergence of *Wolbachia* tropism for the stem-cell-niche in the *Drosophila* testis" PLoS Pathogens. (In Press)

Toomey ME, Panaram K, Fast EM, Beatty C, & Frydman HM (2013) Evolutionarily conserved *Wolbachia*-encoded factors control pattern of stem-cell niche tropism in *Drosophila* ovaries and favor infection. Proc Natl Acad Sci U S A 110(26):10788-10793.

Fast E; **Toomey M**; Panaram K; Desjardins D; Frydman H.M. (2011) *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. Science 18 November 2011: Vol. 334 no. 6058 pp. 990-992.

Toomey, ME, Frydman, HM. Mechanisms of *Wolbachia* tropism to the hub throughout development. (In Preparation)

Frydman HM, **Toomey ME**, Fast E, Simhadri R, Kamath A, Deehan M. Location location, location: *Wolbachia* targeting of tissues during host development. Invited review to be published at the "Annual Review of Cell and Developmental Biology". (In Preparation)

Deehan, M, **Toomey, ME**, Frydman, HM. Identification of stem cells and their niches in the *Culex pipiens* ovary. (In preparation)

POSTERS AND PRESENTATIONS

51st Annual *Drosophila* Research Conference, poster presentation, April 2010.

"*Wolbachia* encoded factors determine differential targeting of stem cell niches"

Kanchana Panaram*, Michelle Toomey*, Eva Fast, Barrett Steinberg, Cathy Beatty and Horacio M. Frydman

6th International *Wolbachia* Conference, Asilomar, CA, poster presentation, June 2010.

"Stem cell niche tropism as a novel mechanism for *Wolbachia* transmission" Michelle

Toomey, Kanchana Panaram, Eva Fast, Barrett Steinberg, Catherine Beatty, Horacio Frydman. *First Prize for Student and Postdoctoral Poster Presentation Competition

52nd Annual *Drosophila* Research Conference, poster presentation, March 2011.

"*Wolbachia* determine differential stem cell niche tropism in the *Drosophila* ovary and testes" Michelle Toomey, Kanchana Panaram, Eva Fast, Barrett Steinberg, Cathy Beatty and Horacio M. Frydman

Biology Department Graduate Student Symposium, oral presentation, May 2012. "Men are messy: *Wolbachia* stem cell niche tropism in *Drosophila* is evolutionarily conserved only in females." Michelle Toomey, Kanchana Panaram, Eva Fast, Cathy Beatty, Horacio Frydman *Awarded 2nd best talk

7th International *Wolbachia* Conference, Oral Presentation, June 2012. “Men are messy: *Wolbachia* stem cell niche tropism is evolutionarily conserved only in females.” Michelle Toomey, Kanchana Panaram, Eva Fast, Cathy Beatty, Horacio Frydman *Awarded best oral communication

Gordon Conference on Tropical Infectious Diseases, Poster presentation and selected for a short talk, February 2013. “*Wolbachia* tropism for stem cell niches is a novel mechanism of symbiont transmission” Michelle Toomey, Mark Deehan, Kanchana Panaram, Eva Fast, Cathy Beatty and Horacio M. Frydman

54th Annual *Drosophila* Research Conference, poster presentation, April, 2013. “*Wolbachia* tropism for stem cell niches is a novel mechanism of symbiont transmission” Michelle Toomey, Mark Deehan, Kanchana Panaram, Eva Fast, Cathy Beatty and Horacio M. Frydman

Biology Department Graduate Student Symposium, oral presentation, May 2013. “*Wolbachia* tropism for stem cell niches is a novel mechanism of symbiont transmission” Michelle Toomey, Kanchana Panaram, Eva Fast, Cathy Beatty, Horacio Frydman *Awarded best talk

8th International *Wolbachia* Conference, Oral Presentation, June 2014. “Mechanisms of *Wolbachia* tropism to the stem cell niche in the *Drosophila* testis.” Michelle Toomey, Horacio Frydman *Awarded 2nd place for best oral communication

TEACHING EXPERIENCE

Teaching Fellow, Boston University, September 2008- December 2009

Taught introductory level biology lab (BI 107/108/118) to students of various levels. Responsible for presenting background material and skills necessary to succeed in the Biological Sciences.

Tutor, Bridgewater State College, September 2006- May 2007

Peer tutor for general Biology and Chemistry for Biology and Chemistry majors

SERVICE

Laboratory Safety Coordinator, Fall 2010-Spring 2014

Responsible for laboratory keeping safety practices, protocols, inventories, and training up to date; Responsible for coordinating safety inspections with supervisor.

Biology Graduate Student Association (BGSA), Executive Committee, September 2010-August 2012 *President September 2011-August 2012

Mission: to provide academic, career building, and recreational activities to the biology community; Organized professional development seminars, faculty and student bio-mixer seminars, and the Graduate Student Symposium

Graduate Student Recruitment, Each spring 2009-2014

Participated in recruitment of new graduate student candidates

Biology Inquiry & Outreach with Boston University Graduate Students (BIOBUGS), Fall 2011

Outreach program designed to encourage local high school students to become excited about science by exposing them to sophisticated scientific equipment, providing interaction with graduate students, and introducing students to a university campus; Volunteer for supervision of high school students during lab experiments

SET in the city, Spring 2010

Outreach program for underprivileged high school girls; Demonstration of *Drosophila melanogaster* mutant flies

Resident Assistant, Bridgewater State College, September 2006- May 2007

For two semesters as an undergraduate, developed social and academic programming for residential community, and acted as student leader through one on one and group interactions with residents and fellow staff members.

RELATED WORK EXPERIENCE

Research technician, Vector Transmitted Infectious Disease Core, NEIDL, January 2010-August 2013

Provide services for the establishment of the Vector Transmitted Infectious Disease Core, including assisting in grant applications; Participated in numerous Biosafety level 4 trainings and simulations, including large scale evacuation simulations

SKILLS & ABILITIES

Laboratory Skills

Trained in Biosafety Level 4 High Containment; cloning; confocal microscopy; DNA, RNA and Protein Gel Electrophoresis; DNA, RNA and Protein Extraction; *Drosophila* genetics; *Drosophila* and *Culex* husbandry; immunohistochemistry; in situ hybridization; microdissection; gel electrophoresis; mosquito husbandry; PCR; qPCR; RNAi; sequencing; transcriptional analysis.

Computer Skills

MatLab, Adobe Photoshop, Slidebook, R, and all Microsoft Office applications.