



The good, the bad, and the ugly: From planarians to parasites

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Contents

1. Seeing the parallels (PAN)	346
2. Schisto happens, too: Becoming a full-fledged parasitology lab (JJC)	350
2.1 It all starts with a fluke	350
2.2 Getting exposed to the parasites	352
2.3 The work begins	353
2.4 Worming our way into new biology	354
3. Transitioning to tapeworms... (PAN)	358
4. Resurrecting a model tapeworm: <i>Hymenolepis diminuta</i> (TR)	359
4.1 An unexpected beginning	359
4.2 It takes a village to "raise" a tapeworm	360
4.3 Good housekeeping matters, even for parasites	362
4.4 Reflections on tool building	365
4.5 Even tapeworms need friends	368
5. Concluding thoughts	369
Acknowledgments	369
References	370

Abstract

Platyhelminthes can perhaps rightly be described as a phylum of the good, the bad, and the ugly: remarkable free-living worms that colonize land, river, and sea, which are often rife with color and can display extraordinary regenerative ability; parasitic worms like schistosomes that cause devastating disease and suffering; and monstrous tapeworms that are the stuff of nightmares. In this chapter, we will explore how our research expanded beyond free-living planarians to their gruesome parasitic cousins. We start with *Schistosoma mansoni*, which is not a new model; however, approaching these parasites from a developmental perspective required a reinvention that may hold generalizable lessons to basic biologists interested in pivoting to disease models.

We then turn to our (re)establishment of the rat tapeworm *Hymenolepis diminuta*, a once-favorite model that had been largely forgotten by the molecular biology revolution. Here we tell our stories in three, first-person narratives in order to convey personal views of our experiences. Welcome to the dark side.



1. Seeing the parallels (PAN)

“What led a planarian lab to start working on parasites?” is the second-most-often-asked question when I meet with trainees during seminar visits and scientific meetings (see Chapter “*Schmidtea* happens: Re-establishing the planarian as a model for studying the mechanisms of regeneration” by Newmark and Sánchez Alvarado for the most-often-asked question). The seeds for our work on parasitic flatworms were planted when I was beginning my work on planarians as a post-doc in Barcelona. As I contemplated returning to the US, but was struggling to find any PIs interested in having me bring planarians to their labs (see Chapter “*Schmidtea* happens: Re-establishing the planarian as a model for studying the mechanisms of regeneration” by Newmark and Sánchez Alvarado), it was beginning to look as if I would need to come up with a plan for a second post-doc. As I started expanding my reading to think about other potential research topics to pursue, I read a small, paperback, introductory parasitology book (Whitfield, 1979) that was one of the few books I brought with me to Spain. This book came to me courtesy of Sean Eddy, a fellow grad student in Boulder, when he was shedding belongings in preparation for his move to the UK for a post-doc. Re-learning the complexities of many parasitic life cycles (which I hadn’t thought about since freshman introductory biology) and thinking about the various ways in which parasites interacted with their hosts convinced me that if I had to abandon planarians, I would study parasitology, instead. Fortunately, I didn’t have to give up on the planarian project, so I wound up not pursuing this plan “B” (at least not for another 15 years or so).

When Alejandro Sánchez Alvarado and I began generating thousands of expressed sequence tags (ESTs) from planarians, we noticed genes that were shared between planarians and parasitic flatworms, but not found elsewhere in the animal kingdom. Our manuscript reporting the initial EST database for asexual *Schmidtea mediterranea* and the development of high-throughput, automated in situ hybridization to identify cell-type-specific markers (Sánchez Alvarado, Newmark, Robb, & Juste, 2002) highlighted some

aspects of the fascinating biology of parasitic flatworms, which infect hundreds of millions of people, and concluded by emphasizing the possibility of using planarians as free-living models for studying their parasitic counterparts. Indeed, Alejandro and I even submitted a patent invention disclosure based on this idea, but it was rejected by the Carnegie Institution's patent attorneys. My lab's paper characterizing ESTs from the sexual strain of *S. mediterranea* (Zayas, Hernández, et al., 2005) returned to this idea, noting that roughly half of the planarian ESTs had homologs in *Schistosoma mansoni*. We also pointed out that the identification of genes encoding signaling molecule receptors in the parasite's genome did not necessarily mean that those receptors were responding to host signals, particularly if those receptors were found in free-living flatworms; thus, it was important to compare the parasites to their free-living relatives before reaching such conclusions.

As the work on planarians continued to develop, I had hoped that researchers studying parasitic flatworms would begin to apply some of the tools we were developing and the lessons we were learning to their own experimental subjects. When it became clear that this wasn't really happening, I then hoped to recruit a post-doc with expertise in parasitic flatworms to pursue this idea. Such a recruit never materialized, so this plan lay dormant for several years.

Meanwhile, our work on planarian germ cell development was progressing well. Graduate student Yuying Wang showed that RNA interference (RNAi) knockdown of the conserved germ cell regulator, *nanos*, resulted in planarians without germ cells (Wang, Zayas, Guo, & Newmark, 2007); she used this knowledge to conduct a non-biased, gene expression-based, functional screen for genes required for germ cell development. Yuying compared gene expression profiles (via microarrays) of planarians with and without germ cells (*nanos* RNAi), then identified RNAi knockdowns leading to defects at distinct stages of male germ cell development. In addition to genes conserved between planarians and mammals, Yuying identified genes that were shared between planarians and schistosomes, but not found in other organisms (Wang, Stry, Wilhelm, & Newmark, 2010); thus, her work ascribed functions to flatworm-specific genes.

Concurrently, post-doc Jim Collins was characterizing the entire complement of peptide hormones/neuropeptides encoded by the planarian genome. These molecules arguably constitute the largest and most diverse class of signaling molecules in animals, and they regulate a wide range of physiological processes from neurotransmission and behavior to feeding and sexual

development. Jim's work integrated genomic prediction of peptide hormones with biochemical/mass spectrophotometric analysis of peptides (in collaboration with Jonathan Sweedler's lab), followed by whole-mount in situ hybridization to map the expression patterns of each peptide hormone gene (Collins et al., 2010). Jim found that knocking down the expression of *prohormone convertase 2* (*pc2*), which encodes an enzyme critical for peptide hormone processing, led to testis regression in sexual planarians, resulting in testes that contained only the initial stages of germ cell development. This phenotype was reminiscent of the testis regression observed after head amputation (Feddecka-Bruner, 1967; Ghirardelli, 1965) and implicated peptide hormones in the systemic regulation of germ cell development. The question was: which peptide hormone(s) mediated this effect on the reproductive system?

Among his collection of 51 genes (which encoded over 200 peptides) Jim was able to find a single peptide hormone-encoding gene (*npy-8*) that was expressed robustly in the nervous system of sexual planarians but whose transcripts were not detectable in asexual worms. Surprisingly, *npy-8* RNAi in mature adults led to testis regression resembling that seen in *pc2* RNAi animals; knocking down *npy-8* during the course of juvenile growth blocked proper development of the reproductive system (Collins et al., 2010). Thus, a single neuropeptide was regulating the development and maintenance of the planarian reproductive system, suggesting an ancient origin for the neuroendocrine control of reproductive maturation (and that planarians also experience puberty!). Beyond its implications for understanding the systemic regulation of germ cell development, Jim identified planarian neuropeptides that had homologs in *S. mansoni* and/or *S. japonicum*, but had yet to be annotated, again demonstrating the utility of such comparative approaches.

As Jim was wrapping up this comprehensive work for publication (please note that I've omitted several other major conclusions of the paper to focus on the topic at hand), he came to an important choice point in his post-doc career: would he continue to study planarian neuropeptides or would he pursue a different biological problem? This question led him to his own deep dive into the literature, starting with Volume 2 of Libbie Hyman's remarkable series of monographs, "The Invertebrates" (Hyman, 1951), which included her overview of the Platyhelminthes. Jim became fascinated by the extreme biology exhibited by parasitic flatworms and by their parallels with planarians (see below). One of the most striking realizations to emerge from his readings was that the pathology of schistosomiasis, which impacts over 200 million people, was almost entirely due to the parasite's prolific reproductive output (Basch, 1991). Female schistosomes produce >300 eggs

per day; blood flow away from the parasite's niche leads approximately half of these eggs to get trapped in host tissues, triggering massive inflammatory responses that can lead to tissue dysfunction and failure. Thus, schistosomiasis is essentially a disease of flatworm reproduction. Jim had just identified a single neuropeptide required for the development and maintenance of the planarian reproductive system; he found that the schistosome genome also encoded this neuropeptide. Could blocking this signaling pathway also switch off the parasite's reproductive system, thereby halting disease pathology and preventing parasite transmission? Here we had this fascinating convergence of a fundamental question of developmental biology with a public health problem of global significance.

As Jim and I discussed the possibility of pursuing this exciting idea ourselves, there were a few major barriers that dramatically increased the activation energy required to get started; all of these barriers were in some way related to the schistosome life cycle (Fig. 1). These blood flukes are obligate parasites of two different animal hosts: a mammalian definitive host (in which

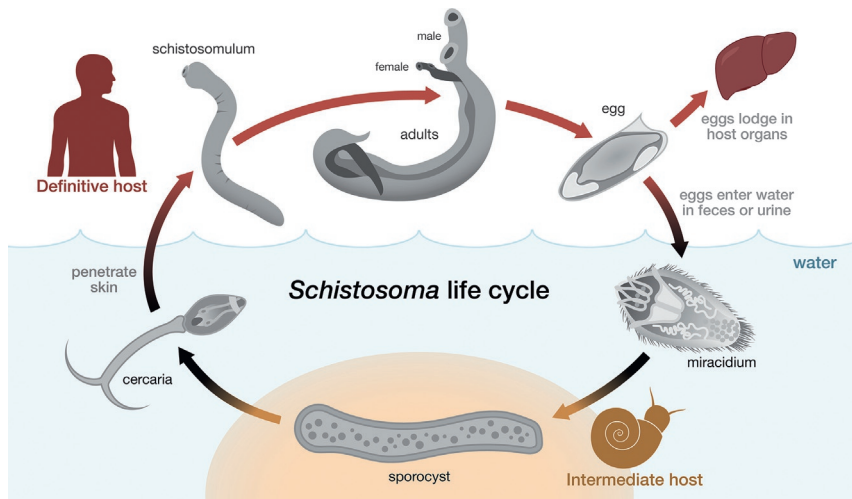


Fig. 1 The schistosome life cycle. Adult parasites lay eggs: roughly half of these eggs get stuck in host tissues, causing disease, whereas the other half are released via urine or feces. Eggs that reach freshwater hatch and liberate miracidia that infect a snail intermediate host. Inside the snail, the miracidia become sporocysts that undergo clonal expansion, ultimately producing cercariae that emerge from the snail to infect a mammalian definitive host. After penetrating the skin of their host, cercariae become schistosomula that migrate through the tissue and into the bloodstream. Once in the portal circulation, the worms develop as either males or females and reproduce sexually.

sexual reproduction occurs) and a snail intermediate host (in which asexual reproduction occurs). Working with the mammalian host (*S. mansoni* can be maintained in the lab in mice) required us to dive into the world of animal care protocols, institutional animal care and use committees, and animal care facilities. Furthermore, working with the snails added the complexity of requiring biosafety level 2 (BSL-2) protocols, approvals, and appropriate lab space. As planarian researchers, we were blissfully unaware of such matters, and were daunted by all of these additional administrative, animal care, and safety burdens.

One day, while still in discussions about whether or how to proceed on the parasitic flatworm front, Jim came charging into my office with a *PLOS NTD* paper by Fred Lewis and colleagues, which described the Schistosomiasis Resource Center (SRC), a National Institutes of Health (NIH)-National Institute of Allergy and Infectious Diseases (NIAID)-funded resource dating back to the 1960s, which was established to facilitate research on these important parasites (Lewis, Liang, Raghavan, & Knight, 2008). It turned out that the SRC was actively maintaining several strains of the major schistosome species infecting humans, along with their different snail intermediate hosts. In addition to offering courses in maintaining the schistosome life cycle, at that time they provided, free-of-charge, schistosome-infected mice and snails to qualified laboratories around the world. After learning about this remarkable resource, within the hour we contacted Fred, who graciously arranged for us to visit his laboratory at the Biomedical Research Institute in Rockville, Maryland to learn how to handle schistosomes.



2. Schisto happens, too: Becoming a full-fledged parasitology lab (JJC)

2.1 It all starts with a fluke

I had long been inspired by Phil and Alejandro's revival of planarians as a model organism (see PAN and ASA chapter, this volume). So, it was exhilarating to have the opportunity to bring schistosomes to the lab. However, as a mid-stage post-doc with a career on the line, this exhilaration was tempered by a fair dose of apprehension. There were many unknowns and no guarantee of success. The biggest unknowns circled around two simple questions: as developmental biologists, with no background in parasitology, could we actually maintain these parasites in the lab and could we ask meaningful and experimentally tractable biological questions in an organism renowned for its experimental intractability?

My trepidation wasn't unwarranted when you consider the worm's life cycle (Fig. 1). This life cycle starts when the parasite's egg reaches freshwater, triggering the release of a ciliated larva, called a miracidium, which despite its simple appearance is quite sophisticated, possessing both an excretory system and a fairly elaborate nervous system. This short-lived larva then has less than 24 h to infect a snail intermediate host. It turns out each schistosome species can only infect specific genera of snails, and thus the parasite's geographic range is not dictated by its definitive vertebrate host but rather the range of the snail intermediate host.

Upon locating the appropriate snail, the miracidium transforms to another larval stage called a sporocyst. The sporocyst is arguably the most critical stage of the life cycle, as it serves as the conduit through which a single miracidium undergoes clonal expansion, thus amplifying the parasite's opportunity to pass to a mammal. This clonal expansion is driven by a small number of undifferentiated cells (germinal cells) that begin to proliferate once the miracidium sheds its cilia and transforms to a sporocyst inside the snail (Wang, Collins, & Newmark, 2013). The germinal cells then enter a phase of asexual embryogenesis, generating so-called daughter sporocysts that migrate to distal parts of the snail. Like their mothers, these daughter sporocysts also contain pools of undifferentiated cells that seed multiple rounds of clonal expansion, generating either new daughter sporocysts or infective larvae called cercariae. Triggered by light, these cercariae wriggle out of the snail tissue and are liberated into the water.

Cercariae use their muscular, forked tail to zip around the water, waiting for the opportunity to infect a vertebrate. Attracted by movement and fatty acids from the skin (Shiff, Cmelik, Ley, & Kriel, 1972), the worms seek out their host, deploy secretions that compromise the epidermis, and break off their tail as they wriggle into the skin. This penetration process occurs within a matter of minutes (McKerrow & Salter, 2002) and the parasites make their way through the tissue before entering the vasculature. The worms then make several passes through the circulation before taking up residence in either the portal vasculature or the venous plexus of the bladder. Once at their final destination, the worms mature to adulthood as either males or females. The male schistosome is large and muscular and possesses a groove on his ventral surface (gynecophoral canal) that he uses to grasp the long, slender female.

Not only do schistosomes have the distinction of being the only non-hermaphroditic, dioecious (i.e., separate male and female sexes) group of flatworms, but this close physical contact between the male and female

worms is absolutely essential for female sexual development. Female worms grown in hosts without males are small and their reproductive organs are present as undeveloped primordia (LoVerde, 2002; Severinghaus, 1928). Upon pairing with a male, the female perceives a yet-to-be defined signal, initiating the development of the sexual organs and ultimately egg production. The eggs laid by the sexually mature female are then washed away into the blood with the goal of reaching the outside world. Roughly half of these eggs move from the blood to the intestine (or bladder for urogenital schistosomiasis) to be passed to the environment via feces (or urine). The other half, however, become lodged in host organs inducing inflammatory lesions that are the sole driver of morbidity and death due to schistosome infection (Pearce & MacDonald, 2002).

2.2 Getting exposed to the parasites

As Phil noted above, our journey to schistosomes moved from the “hypothetical” phase to the “ok, we are doing this” phase when we contacted Fred Lewis. Fred, a veteran of the schistosome field had spent his career optimizing every aspect of schistosome husbandry (Lewis, 2001). One of the main challenges of schistosome maintenance wasn’t keeping the worms alive, but rather growing and maintaining the sometimes-fastidious snails. Since the SRC propagated the three major species that infect humans (*S. mansoni*, *S. japonicum*, and *S. haematobium*) that meant they had to grow three varieties of snails, each presenting their own challenges. In fact, the SRC is the only facility I am aware of that actively maintains the notoriously demanding *Oncomelania* snails that serve as the intermediate host of *S. japonicum*. These snails will not eat lettuce like other snail hosts and prefer rather to eat single-celled critters (e.g., diatoms and algae). Fred and his colleagues developed a methodology to grow *Nostoc* (cyanobacterium) on mud, lime and chicken manure and use it as a food source for *Oncomelania*. In fact, the SRC had a dedicated “mud room” that, well, was a room full of (you guessed it) mud they routinely collected from a specific locale in Maryland.

Fred had studied these snails so intently, that he even noted that these snails often harbor commensal rotifers and that some rotifer species could actually make a diffusible substance that paralyzes cercariae, preventing them from infecting mice (Stirewalt & Lewis, 1981). So as part of the SRC snail maintenance protocols, folks routinely use a Waterpik (yeah, the one for your teeth) to blast rotifers off of snail shells. (Side note: It turns out Fred did quite a bit of work in the late 1980s trying to find the rotifer-derived

compound only to have funding for the work expire and his progress go unpublished. A few decades later, Fred shared his unpublished observations with Phil and me. This topic became the subject of Phil's 2011 summer "back to the bench" project, culminating in the elucidation of a novel, rotifer-derived alkaloid with schistosome paralytic activity (Gao et al., 2019).

After a few days with Fred at the SRC learning the ins and outs of snail maintenance, how to get cercariae from snails, and expose mice to the parasites, Phil and I got our first real "exposure" to parasitology when it came time to recover worms from mice. It was as impressive as it was gruesome. For a couple of guys who worked on invertebrates most of their careers, this was an eye-popping experience. It started with euthanasia of about 50 schistosome-infected mice and these expired mice made their way into a large sink full of water to mat down their fur. I don't recall in my career ever seeing a single dead mouse, much less a whole sink full. From here, the scissors came out, first for removing the skin and then for exposing the contents of the peritoneal cavity. By precisely severing the portal vein under the liver and plunging a needle into the aorta, the worms were perfused from the blood into a large bloody container beneath, with the worms often liberated as large boluses visible to the eye. Now collecting the worms wouldn't be enough to close the life cycle, since eggs and miracidia are required to infect snails. This requires the egg-riddled livers. So, Fred's team would retrieve the livers, puree them in a Waring Blender, pass the material through a series of mesh sieves, and then put what remained into freshwater. Once in freshwater, the miracidia are liberated from the eggs to infect snails, thus closing the circle.

2.3 The work begins

Following our brief visit to the SRC, we returned to Illinois and got to work. Unfortunately, before the parasite work could start we had to convert parts of a planarian developmental biology lab into a space suitable for both animal work and for handling BSL-2 pathogens. The investment in equipment was minimal, but the investment in time navigating both IACUC and the Institutional Biosafety Committee was considerable for uninitiated chaps like Phil and me. Fortunately, Fred was able to put us into contact with David Williams, a schistosome biochemist whose lab was just a couple of hours away at Rush University in Chicago. We established a collaboration with David, showing him the ropes on planarian husbandry while he provided us support for our nascent schistosome efforts, including sharing details of his animal and biosafety registrations.

In the intervening eight or so months between committing to working on schistosomes and actually receiving parasites in the lab, Phil and I spent a lot of time going through the literature. One thing that frustrated us in particular, was we had no idea what these parasites looked like on the inside. Certainly, there were volumes of beautiful ultrastructural studies from the 60s, but few studies using modern confocal microscopy to visualize the worm's tissues and organ systems. This is at a time when confocal images of planarians had made their way to countless journal covers, yet, we couldn't find a single image of a schistosome stained with something as simple as DAPI. So, our first priority once we finally got worms in the lab was to throw them in formaldehyde and then pull things out of the freezer to see what stuck. Together with another post-doc, Ryan King, who was interested in the parasite's protonephridial system, we threw basically every fluorescent stain, lectin, and antibody we could dig out of the freezer on various stages of the worms and then spent hours a day monopolizing the confocal microscope. These efforts, which spanned over just a couple of months, paid off, defining a series of beautiful markers to visualize the reproductive, protonephridial, and nervous systems as well as the worm's many types of secretory organs. As we wrote this "atlas" detailing the worm's organ systems (Collins, King, Cogswell, Williams, & Newmark, 2011), there was some trepidation about the reception the work would receive in the field. I have certainly heard of outsiders wandering into established fields and being met with a great deal of hostility. However, we were pleasantly surprised that not only did the paper fly through peer review, but we received a number of encouraging emails from people welcoming us to the field. In fact, we have always found folks in the schistosome community to be exceptionally collegial and welcoming to newcomers.

2.4 Worming our way into new biology

Armed with a collection of cell-type-specific markers and a newly adapted protocol for whole-mount in situ hybridization that we developed alongside David Williams' group (Cogswell, Collins, Newmark, & Williams, 2011; Collins et al., 2013), 2 years in we were finally ready to explore some biology. Based on Hyman's descriptions, it was clear that parasitic flatworms had all sorts of extreme biology that was strangely reminiscent of what occurs in their planarian relatives. Tapeworms can grow meters in length by perpetually adding segments to their body and as detailed above, schistosomes (and all other trematodes) asexually reproduce in their intermediate hosts by

undergoing endless rounds of asexual embryogenesis. Importantly, all of this extreme biology appeared to be driven by cells that resembled the stem cells (neoblasts) that give the planarians their ability to regenerate. Given this, Phil and I were taken with the idea that during the course of evolution these parasites simply repurposed the tricks of their free-living, planarian-like ancestors to thrive as parasites.

One observation that Phil and I were interested in tackling first was the intriguing observation that schistosomes can live for decades inside their mammalian host. In the literature there is no shortage of medical case studies in which a person moves from a schistosome-endemic region to a non-endemic country as a child to find out as an adult that they have schistosomes alive in their bloodstream, laying eggs. Remember, the adult parasites that lay eggs do not replicate in the blood, thus, these adult parasites are the very same worms that have lived with these adult patients since childhood. The literature describes some extreme examples in which people are infected with schistosomes 30+ years after leaving an endemic region (Harris, Russell, & Charters, 1984; Hornstein et al., 1990; Payet et al., 2009)! So what is the mechanism underlying this longevity?

To Phil and me the answer was obvious: they must use neoblast-like cells to maintain their tissues in a manner similar to how the planarian ensures its immortality. However, examination of the literature found scant evidence that schistosomes (or any other trematode) possessed any proliferative cells outside their reproductive organs. It was also clear that no one had ever really looked for such cells systematically. So we did the most straightforward experiment we could imagine: culture the worms in vitro in tissue culture media and pulse them with the thymidine analog EdU to determine if they possessed any proliferative cells in their somatic tissues. Excitingly (but not totally unexpectedly) there were loads of EdU-labeled cells scattered throughout the worm's soma (Collins et al., 2013). We went on to show that these neoblast-like cells were true stem cells capable of self-renewal and differentiation. We also pulled a trick from the planarian biology grab bag to use irradiation to kill the stem cells and then perform transcriptional profiling to define neoblast-specific markers. Not only did this provide a description of the cells on a molecular level, it provided an avenue to begin conducting functional experiments to define genes required for stem cell function.

As of 2012 when we started exploring these neoblast-like cells, RNAi had already been shown years prior to function in in vitro-cultured schistosomes across a variety of life-cycle stages (Boyle, Wu, Shoemaker, & Yoshino, 2003; Skelly, Da'dara, & Harn, 2003). However, anecdotal

observations and published reports (Krautz-Peterson, Bhardwaj, Faghiri, Tararam, & Skelly, 2010; Štefanić et al., 2010) suggested that the effects of RNAi were variable, with some genes totally non-susceptible to the effects of double-stranded RNA (dsRNA). This was unsurprising since this is the case in a variety of systems. What was more concerning was that there was little evidence that RNAi could generate reproducible phenotypes, particularly in the adult stage of the life cycle. If mRNA silencing was robust, then where were the phenotypes?

Working from our foundation in planarian biology, we reasoned the troubles were twofold. First, it was clear from many published reports that phenotypes were scored shortly after dsRNA treatment (usually 1 week). However, in planarians very few phenotypes could be seen in this timeframe. Since planarians have massive amounts of tissue turnover (and thus protein turnover) relative to schistosomes, it seemed unlikely that dramatic phenotypes could be observed in such a short interval. This was an easy fix: just wait longer. The second issue was that most studies only examined a single gene at a time, and since the effects of RNAi varied from gene to gene, one would likely have to examine multiple genes involved in a given process to yield a phenotype. What was the barrier to doing this? Well, to achieve optimal mRNA knockdown, worms had to be cultured in high concentrations of dsRNA in several mL of media (Krautz-Peterson et al., 2010; Krautz-Peterson, Radwanska, Ndegwa, Shoemaker, & Skelly, 2007; Štefanić et al., 2010). Since the field had relied on either expensive “high-yield” dsRNA synthesis kits (Štefanić et al., 2010) or commercially synthesized siRNAs (Krautz-Peterson et al., 2007) for their experiments, it was cost prohibitive to examine more than a few genes at a time.

So we took another trick from the planarian playbook. At the time, many in the Newmark lab had migrated from using bacterially expressed dsRNA for RNAi in planarians to in vitro-synthesized dsRNA made using a super-cheap, homemade “high-yield” recipe (Rouhana et al., 2013). This meant we could overcome the sampling error that in part obscured phenotypes and quickly screen tens, if not hundreds, of genes in a single experiment. So, with these tweaks in place, we cloned cDNAs from ~30 of our neoblast-expressed gene list, treated worms with dsRNAs, and examined the worms by EdU labeling about 2½ weeks later. Not only did we observe that loss of several canonical cell-cycle regulators caused stem cell maintenance defects, but loss of one of the worm’s FGF receptors caused a dramatic reduction in stem cell number (Collins et al., 2013). I had been skeptical about whether we would be able to ask the types of developmental

questions that drew us to the field in the first place (see question #2 above), but with the results of these RNAi experiments it was clear we had a real honest-to-goodness system to study new and interesting biology.

Buoyed by our studies on schistosome stem cells, we felt empowered and wanted to add another experimental arrow to our quiver. In particular, since these are obligate parasites, studies during *in vitro* culture can only elucidate so much of the parasite's biology. For instance, the reproductive biology of the parasite represents not only amazing biology but a key driver of disease pathology. Unfortunately, at the time culture conditions were insufficient to maintain sexual reproduction for more than a few days outside the host (we have since addressed this (Wang, Chen, & Collins, 2019)). However, there weren't just technical limitations to *in vitro* culture, there are simply many critical interactions between the worms and their host that cannot be replicated in a dish. For example, how is it that these worms thrive on the front lines of the host immune response for decades without being cleared? To this end, we wanted to develop a method that would allow us to experimentally manipulate worms in the context of their host; without the convenience of germline transgenics, we had to think outside the box a bit.

In the summer of 2013 when preparing materials for my upcoming job hunt, I came across a paper from 1976 by Donato Cioli, a schistosome researcher who had developed an approach to surgically transplant schistosomes from one host to another (Cioli, 1976). I reasoned that we could retrieve worms from a host, manipulate them *in vitro* (e.g., RNAi treatment), then transplant them to a new host and examine what happens to the worm. As luck would have it, Donato was still working on schistosomes and had come across our atlas paper. He reached out to us for our input with some of his ongoing work on the mechanism of action of the anti-schistosomal drug Praziquantel. So, I contacted Donato and inquired about the technique he developed almost 40 years prior. Within 24 h I received a response complete with a highly detailed protocol of the procedure, including catalog numbers of specific types of custom needles he used to inject worms and his preferred anesthetic. The protocol entailed anesthetizing the recipient mice, pulling the intestines through a small incision, injecting the worms into a specific vein (cecal vein), stopping the bleeding with hemostatic gauze, reinserting the intestines, and closing the incision with sutures.

Although Donato was a trained physician, the most complicated surgery I had ever done involved a planarian and a razor blade. So I was relieved that in the post-script of his email he offered to host us in his lab

and teach us the technique. So I talked to Phil and, although he couldn't travel because of NIH study section duties, he gave me the green light to visit. Oh, did I mention that Donato lived in Rome? So, I packed my bags and traveled to sunny Italy for 3 days of training in Donato's lab at the Institute of Cell Biology and Neurobiology in Monterotondo outside of Rome. Aside from being an outstanding source of knowledge about schistosome biology he was an astounding teacher and I left Italy having successfully transplanted schistosomes into several mice. (Side note: Not only was Donato an amazing host and teacher, but perhaps the greatest tour guide of Italy anyone could imagine. He knew so much about Italian and Roman History and he actually spent his time outside of the lab as an archeologist exploring ancient Roman aqueducts. Also, I didn't even bring Phil a t-shirt, as he likes to remind me.)

My time at Illinois outfitting parts of Phil's lab for schistosome work set the stage for starting my own lab at UT Southwestern. With this relatively modest tool kit we've been able to dive deeper into schistosome stem cell biology (Collins, Wendt, Iyer, & Newmark, 2016; Wendt et al., 2018, 2020), define new regulators of sexual development (Wang et al., 2019; Wang & Collins, 2016), determine the functions of schistosome genes in vivo (Collins & Collins, 2016; Wang et al., 2020), and even leverage these tools to prioritize therapeutic targets (Wang et al., 2020).



3. Transitioning to tapeworms... (PAN)

In the summer of 2012, Jim and I went to the Gordon Conference on Host-Parasite Interactions, the first parasitology conference either of us had attended. I had the opportunity to present Jim's work showing that adult schistosomes have stem cells, just like their planarian cousins. I was feeling quite proud of how well the work had progressed and how the idea of approaching the study of these parasites from a developmental perspective had yielded new insights into their biology. When my talk ended, the first question came from Jayne Raper, who asked if we had also characterized such stem cells in tapeworms. I wound up saying something to the effect of, "I wish we had, but this was already quite a bit of work: it took us several years to get to this point, and as interesting as tapeworms are, we simply haven't looked at them yet." In our earlier discussions of the amazing biology of parasitic flatworms, Jim and I had talked about the seemingly limitless growth of tapeworms and how it was driven by stem cells, but we had our hands full with the schistosome work. Nonetheless, Jayne's question gnawed at me and I kept tapeworms at the back of my mind.

That November, I was invited to speak at the graduate student–organized departmental retreat for the Molecular Biology Program at the University of Colorado School of Medicine. Dick Davis was one of the faculty members attending the retreat; Dick and I had corresponded a decade or so earlier based on his previous work on trans-splicing in schistosomes (Davis, Hardwick, Tavernier, Hodgson, & Singh, 1995; Davis & Hodgson, 1997; Rajkovic, Davis, Simonsen, & Rottman, 1990) when my lab had found similar trans-splicing in planarians (Zayas, Bold, & Newmark, 2005), but we had never met in person. Dick was now doing fascinating work on chromatin diminution in *Ascaris*, bringing genomic tools to bear on the classic observations of Boveri, who described the loss of chromatin in somatic embryonic cells while cells of the germline retained their full chromosome complement (reviewed in Wilson, 1937). I wanted to learn more about this fascinating work, so made a point of tracking him down between sessions. During the course of our discussion, I learned that Dick studied the tapeworm, *Hymenolepis diminuta* for his Ph.D. When I told him that I was interested in looking at tapeworms to explore the similarities between their stem cells and the neoblasts from planarians and schistosomes, Dick graciously volunteered to help get us started with the life cycle. Soon enough, we would come knocking at his door.



4. Resurrecting a model tapeworm: *Hymenolepis diminuta* (TR)

4.1 An unexpected beginning

I arrived in the Newmark lab with every intention of being a good planarian biologist, ready to elucidate mechanisms of regeneration in these remarkable flatworms. Regeneration had long fascinated me and was the subject of my college admissions essay but I took somewhat of a detour in graduate school to study embryogenesis in *Xenopus*. I reasoned that a foundation in developmental biology would be a valuable asset in the study of regeneration and that has certainly remained true. As a newly minted Ph.D., I thought I was finally executing my master plan. Little did I know that there were more twists and turns ahead.

Regeneration is actually a common occurrence across most phyla, though the range of regenerative ability varies widely; many species can regenerate cells/tissues to a limited extent, while far fewer species exhibit whole-body regeneration (Bely & Nyberg, 2010). What brought me to the Newmark lab was its history working with both a champion specimen capable of regenerating both soma and germline—*S. mediterranea*, while also

tapping the diversity of the flatworm phylum in which regenerative ability spans the gamut. Using a multi-species approach, one can probe why some animals can regenerate while others cannot. I was particularly struck by previous work in the lab from James Sikes who showed that *Procotyla fluviatilis*, which normally cannot regenerate a head from a tail fragment, could be coaxed to regenerate its head by simply reducing Wnt/ β -catenin signaling levels (Sikes & Newmark, 2013). The Newmark lab was also making numerous discoveries about the parasitic blood fluke *S. mansoni*; while this species does not regenerate it does exhibit prolific stem cell-driven reproductive output with major implications for the pathology caused by these parasites, as described above. I was excited to work in this veritable zoo of a lab and yet I was unprepared to sit in Phil's office on my first day and hear him ask, "Have you ever thought about tapeworms?" I confessed, I had not. Without exerting any pressure, Phil suggested I just do some reading and see what I thought.

The flood gates opened. Why *hadn't* I thought about tapeworms? They are cousins to planarians that seem to do nothing but grow, often to enormous lengths! As part of their normal life cycle, many tapeworms have the ability to shed and regrow their body! Surely, these are the kinds of "monsters in our midst" that scientists should take advantage of to gain a richer understanding of stem cell and regenerative biology.

4.2 It takes a village to "raise" a tapeworm

As parasites that infect humans and livestock, causing disease and devastating economic burdens, tapeworms have long been a small but persistent subject of scientific research. There are international groups in pursuit of better diagnostic, treatment, and preventative options to eradicate the scourge of these parasites (e.g., The Cysticercosis Working Group in Peru). Could we as developmental biologists bring something to the table? Our approach was to try and uncover fundamental workings of tapeworms both to make basic biological findings and to expose vulnerabilities that can be exploited.

Naturally, most tapeworm research was focused on pathologically relevant species (primarily from the Taeniidae family) that cause cysticercoses and echinococcoses, devastating diseases in which tapeworm larval cysts invade host organs, including liver, lung, and brain (Brutto, 2012; Eckert & Deplazes, 2004). Recently, there has been growing recognition that like the adult tapeworms, the larval cystic forms maintain stem-cell-like populations that are

sometimes called “germinative cells” (Koziol, Rauschendorfer, Rodríguez, Krohne, & Brehm, 2014; Orrego et al., 2021). Thus, understanding tapeworm stem cell regulation has potential for translational relevance (Brehm & Koziol, 2014). Despite all this, our understanding of both stem cells and regeneration in tapeworms was rudimentary at best. Furthermore, the field as a whole suffered from a decided dearth of molecular data, though that was beginning to change through the contributions of Klaus Brehm (Universität Würzburg, Germany), Estella Castillo and Uriel Koziol (Universidad de la República, Uruguay), Pete Olson (The Natural History Museum, United Kingdom) and others. Thus, we reasoned that we could leverage our experience with planarians to gain a molecular understanding of regeneration and stem cell regulation in tapeworms. What we needed was to establish a tapeworm laboratory model amenable to modern molecular experimentation.

How does one choose a model species? My experience teaches me that it is some combination of previous knowledge, intuition, and a healthy dose of luck. There are more than 4000 species of tapeworms and counting (Caira & Jensen, 2017). The majority of tapeworms require host species that would be daunting to work with (e.g., sharks, pigs, humans). For a laboratory model from which we could easily obtain adult worms, we prioritized rodent tapeworms. This quickly narrowed the list down to four popular species from the genus *Hymenolepis*: *H. diminuta*, *H. citelli*, *H. microstoma*, and *H. nana*. Each had their pros and cons. *H. nana* is the most common human tapeworm and thus has direct translational relevance but also posed additional biosafety concerns. *H. microstoma* had a published genome (Tsai et al., 2013), but is an atypical tapeworm that resides in the bile duct instead of the intestine. Furthermore, the Wellcome Sanger Institute was embarking on an ambitious project to sequence dozens of helminth genomes, making available a wealth of genomic data for many species (Holroyd & Sanchez-Flores, 2012). However, to be perfectly honest, the decision was not an agonizing one. As we wanted a good laboratory model, it made sense to choose the species that was most extensively studied in the lab and a clear winner emerged: *H. diminuta*. We were astonished to find a veritable tome numbering 747 pages called “The Biology of the tapeworm *Hymenolepis diminuta*” edited by Hisao P. Arai and published in 1980 (Arai, 1980). This book capped decades of beautiful biochemistry, light and electron microscopy, *in vitro* as well as *in vivo* manipulations, and more. How sad then that after 1980 almost all research on this and other rodent tapeworms

seemed to cease. Why? Anything I say would be mere speculation from a newcomer. It was probably a combination of changing funding priorities, the difficulty of establishing molecular methods on such complicated animals, and the funneling of resources into an ever decreasing number of organisms. To me, I felt both humbled and excited by the chance to revitalize this work and bring *H. diminuta* back to the bench, having the advantage of previous work by outstanding experimentalists from a different era. I personally leaned heavily on the work of Clark Read, Marietta Voge, Chauncey Goodchild, John Ubelaker, Larry Roberts, Richard Lumsden, Robert Specian, and Teresa Sulgostowska, just to name a few.

With Phil's agreement, I cast my lot with *H. diminuta*. Phil then revealed that we were not alone, and relayed to me how Dick Davis had offered his expertise should we need it. As Phil described above, Dick had long since moved on from tapeworms to an even more disgusting parasite, *Ascaris* (Google image search, dear reader, at your peril). In an act of sheer generosity, Dick got his laboratory set up with rats and infected beetles, hosted me in his lab and home, and taught me how to propagate the life cycle and handle these worms. For this, he has my heartfelt and unending gratitude. Up to this point, all my knowledge was from the page and now I was able to touch and see my first tapeworm. I named her Aurora in honor of Aurora, Colorado, where we stood.

4.3 Good housekeeping matters, even for parasites

Back in the Newmark lab, I was now all set to get *H. diminuta* established. It was not difficult to source—infected beetles are actually sold by Carolina Biologicals as they are still used in parasitology classrooms across the country. The life cycle (Fig. 2A) is extraordinarily simple to maintain. The mealworm beetle *Tenebrio molitor* ingests embryonated eggs from infected rat feces and unwittingly plays host to the larval stages of *H. diminuta*. Larvae in the gut can burrow into the hemocoel, where larval development concludes in 2 weeks. In the hemocoel, a transformation occurs in which the anterior end forms structures that will persist in the adult (such as the suckers) and this region becomes encapsulated into a cysticercoïd by the rest of the larval tissue. In essence, the cysticercoïd forms a protective pouch around a dormant juvenile tapeworm, which can remain in suspended animation for the life of the beetle. Thus, batches of infected beetles can be easily maintained in the lab for months in a bed of oats with moisture from carrots as a water source.

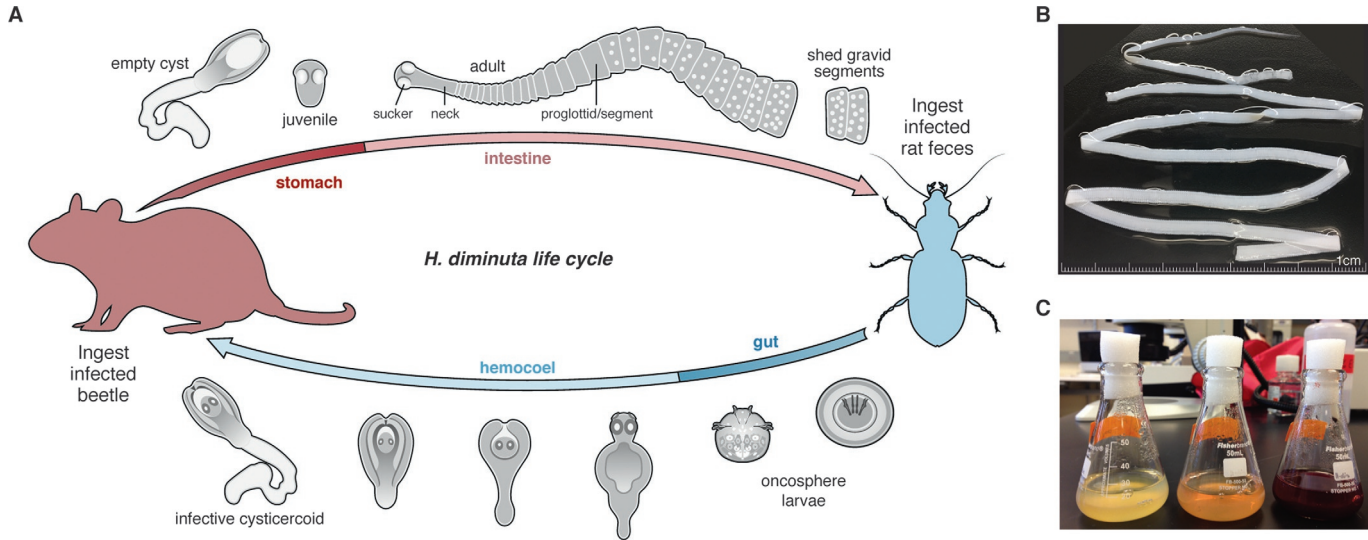


Fig. 2 In vivo and in vitro cultivation of *H. diminuta*. (A) Two hosts are used to complete the *H. diminuta* life cycle: mealworm beetles host larval development from oncosphere to infective cysticeroid and rats host the adult stage through sexual maturity. Each adult forms a head, neck, and strobilated body made of thousands of segments/proglottids. Each mature segment is hermaphroditic, can cross-fertilize, and develop to gravidity. Through apolysis, gravid segments are shed and excreted with the rat feces, which can be consumed by a beetle to complete the life cycle. (B) An adult *H. diminuta* at 20 days old arranged from anterior (top) to posterior (bottom). (C) Adult stages can be grown in vitro using biphasic cultures. Shown are flasks with agar that was enriched with either plasma, serum, or blood (preferred), overlain with Hanks Balanced Salt Solution. Amputated tapeworm fragments containing the neck can grow and regenerate using this in vitro culture system in a hypoxic chamber.

To infect rats with *H. diminuta*, I dissect open the infected beetles and administer 1–500 cysticercoids to rats via oral gavage. The rat's stomach acids and enzymes then permeabilize the cysticercoid and bile triggers excystment of the juvenile tapeworm. At this point, the tapeworm measures 100–200 μm in length and uses its suckers to latch onto intestinal microvilli where it will grow to reproductive maturity and reach an equilibrium length of $\sim 60\text{ cm}$ in 38 days (Chandler, 1939; Roberts, 1980) (Fig. 2B). Adult *H. diminuta* has a head/scolex at the anterior-most end followed by an unsegmented neck (germinative region), which serves as a growth zone from which thousands of segments/proglottids bud (Fig. 2A). Each segment in these hermaphrodites is fated to form all the male and female reproductive structures. Within 2 weeks, *H. diminuta* reaches sexual maturity and can mate with itself or with another worm. The fertilized eggs become deposited in the uterus along with a yolky vitelline cell that forms extraembryonic structures to protect the developing embryo. However, development cannot proceed through larval stages in the rat host. In the wild, *H. diminuta* undergoes apolysis, in which posterior fragments containing gravid segments are released with the rat excrement and can be consumed by an unsuspecting beetle or other similar insects. In the laboratory, I can simply amputate 4–10 cm of adult posteriors, smash them with a little apple sauce on filter paper, and feed the embryo-laden mixture to starved beetles. And thus, the life cycle is completed. Adult *H. diminuta* causes no pathology to its rat hosts and up to 10 mature tapeworms can reside in the rat intestine for the life of the rat. As a former frog embryologist with no parasitology chops, the simple and affordable husbandry involved in this system was a great attraction.

With the guidance I received from Dick, I successfully established a colony of *H. diminuta* in the Newmark lab. I remember running through the hallway toward Phil's office exclaiming "I have tapeworms!" Phil rightfully noted that such a statement rarely came with the level of exuberance I was displaying. I decided to keep my first mature tapeworm as a demo worm that I could show to visitors. I named her Bathsheba (after the tenacious heroine of Thomas Hardy's "Far From the Madding Crowd": Bathsheba Everdene). She has been a useful communication aid that I have now shown to visiting speakers, prospective graduate students, school kids of all ages, and even my wedding guests. As exciting as all this was, the hard part had just begun.

4.4 Reflections on tool building

With no molecular tools established for *H. diminuta*, tool building was an immediate priority. From previous experience, we had learnt that fluorescently conjugated lectins and many common generic antibodies could preferentially label flatworm tissues and allow us to visualize features such as the osmoregulatory canals and tubules, flame cells, gonads, musculature, neurons, and domains of the tegument (skin) (Collins et al., 2011; Rozario & Newmark, 2015; Zayas, Cebrià, Guo, Feng, & Newmark, 2010). These observations confirmed many previous cell-biological findings that had been made by light and electron microscopy (Roberts, 1980) and gave us an appreciation for the complex structures that are continually formed throughout adult development. This was a good way to “get our hands wet” with something we had good reason to suspect would work and would give us a resource we could apply to many future experiments.

Though tool building is obviously necessary, one could get dragged down trying to perfect methods. It was really important that we forged ahead with the dual priorities of establishing methodologies and testing hypotheses. We immediately focused on elucidating regenerative competence in *H. diminuta* using amputation and to describe genetic regulators of stem cells that drive growth and regeneration. To this end, the tools we needed were clear. We prioritized assembling a transcriptome and establishing RNA in situ hybridization, as this would allow us to clone candidate genes of interest and assay gene-expression patterns. We collaborated with Jianbin Wang from Dick Davis’s lab for the transcriptome assembly, which was a combination of de novo and map-based assemblies because at the time, the *H. diminuta* reference genome was highly fragmented. We decided against sequencing the genome ourselves reasoning that genome assemblies for helminths would continue to improve thanks to other researchers with more relevant expertise and indeed there is now a much improved *H. diminuta* genome assembly (Nowak et al., 2019).

For in situ hybridization, we relied on a wealth of previous experience in the Newmark lab but were also generously aided by Pete Olson who shared his working protocol for *H. microstoma*. These advances allowed us to perform RNA sequencing and identify putative stem cell markers by comparing transcriptomes of wild-type tapeworms to tapeworms that were first depleted of stem cells using irradiation (Rozario, Quinn, Wang, Davis, & Newmark, 2019). We were able to gain a molecular description of transcripts enriched

in subpopulations of dividing cells, which include stem cells. It is worth noting that though our work benefits strongly from comparison with planarians, parasitic flatworm stem cells have unique characteristics: key stem cell genes like *piwi*, *tudor*, and *vasa* have been lost from the genomes of parasitic flatworms (Tsai et al., 2013). Thus, unbiased discovery of stem cell markers is also necessary and we are currently pursuing single-cell sequencing of stem cells in *H. diminuta* to capture transcriptional heterogeneity within the stem cell population.

To move this model away from being purely descriptive and into the realm of functional biology, it was crucial to establish loss-of-function methods. For this to be feasible, we first needed to grow these parasites in vitro. Admittedly, one of the main reasons we chose *H. diminuta* in the first place was that all stages had been grown in vitro before, though there were multiple different protocols, and no one had performed these experiments in over 30 years. We concentrated on the adult stage. Most previously described methods grew these worms in a hypoxic, biphasic culture composed of a supplemented nutrient agar that was overlain with a physiological saline in which the worms could reside. Working mostly from papers by Everett Schiller, Larry Roberts, Faith Mong, and Robert Turton (Roberts & Mong, 1973; Schiller, 1965; Turton, 1972), we tried supplementing with blood, serum, or plasma from numerous animal sources, as well as yeast and liver extracts (Fig. 2C). We also varied the saline solutions and glucose concentrations. In truth, many conditions worked to some extent though there was a great deal of variation.

I decided to set a “gold standard.” I wanted a condition in which worms could grow to gravidity in vitro, at which point I would take the tissue with embryonated eggs and use that to complete one life cycle (in both beetle and rat hosts) and recover a fully mature adult tapeworm. The winning combination was defibrinated sheep blood-agar with 4 g/L glucose-supplemented Hanks Balanced Salt Solution. The mature tapeworm we recovered was a milestone indeed. I named her Cara (for “cara mia,” or “my beloved” in Italian. I trust my naming pattern is emerging).

It is worth noting that while I was testing different culture conditions, I was simultaneously performing amputation experiments to test regeneration competence of *H. diminuta*. While this doubled or tripled the number of conditions tested, it was also highly valuable. First, we were able to gain experimental insights while building tools. We were able to uncover that *H. diminuta* regenerates in a regionally limited fashion: the neck is necessary and sufficient for segment regeneration, whereas the head and body are not

regeneration competent. However, regeneration from the neck is finite: persistent regeneration requires the presence of the head (Rozario et al., 2019). Continuing studies with head amputations are revealing that the head either directly or indirectly regulates the microenvironmental signals that are necessary for segment regeneration to occur. Second, doing these kinds of amputation experiments concurrently with varying the culture conditions allowed us to identify the condition that was most permissive for the kinds of experiments we were interested in. Though many experiments then had to be repeated and standardized later, we gained a tremendous amount of confidence and excitement that *H. diminuta* was going to be a worthwhile and interesting model to pursue.

The next holy grail was RNAi. All parasitic flatworms belong to a monophyletic clade called the Neodermata, which refers to a “new skin”: the tegument (Tyler & Tyler, 1997). The tegument is a single cell that encompasses the entire worm exterior and is connected to thousands of nuclei within cytons that make thin cytoplasmic bridges to the shared cytoplasm at the exterior. This unique structure is used for communication and nutrition as it forms the interface with the tapeworm’s environment (Lumsden, 1975; Lumsden & Specian, 1980). Tapeworms have evolved to completely rely on the tegument for nourishment and have entirely dispensed with their digestive system; they have no mouths and no guts. Thus, one would think that they are tailor-made for RNAi and one could simply soak them in double-stranded RNA (dsRNA). This strategy works reasonably well in blood flukes (Wang et al., 2020), which was encouraging to us. However, unlike tapeworms, flukes have both a tegument and digestive tract, enabling two routes for dsRNA uptake. All efforts to soak *H. diminuta* in dsRNA (with carriers, with permeabilization agents, in different volumes and concentrations, using different sizes of dsRNA, etc.) have failed to produce robust gene knockdown. Thankfully, my years working on *Xenopus* came to my aid and I was able to microinject dsRNA into the rather finicky and narrow tapeworm necks to induce RNAi. Consequently, we were able to establish an RNAi paradigm to identify functional regulators of stem cells and regeneration (Rozario et al., 2019). Microinjecting is laborious, time consuming, and relies on “user ability” and thus requires more careful experimental design (additional controls, blinded experiments, and large sample sizes). This is yet another example of how we established methods that are “good enough” for the purpose of immediately testing hypotheses, but with future troubleshooting, these methods will undoubtedly become easier, more high-throughput, and more robust.

These and other experiments began to reveal that though *H. diminuta* could only regenerate from its neck, we could not identify a neck-specific stem cell population that explained this behavior. In fact, cells from both regeneration-competent and regeneration-incompetent regions display collective pluripotency and can rescue viability and regeneration in irradiated/stem cell-deficient tapeworms as long as these cells are transplanted into the neck (Rozario et al., 2019). It is becoming clear that microenvironmental signals in the neck play pivotal roles in regulating regeneration and/or stem cell behaviors. Clearly there is much to learn from these enigmatic creatures.

4.5 Even tapeworms need friends

However enthusiastic we may be, all would be for naught if we could not generate interest among our colleagues and the greater scientific community. Perhaps we are fortunate that both parasitologists and developmental biologists work under big tents. The study of parasitology involves cell and molecular biology, microbiology, immunology, physiology, chemistry, epidemiology, and ecology. Similarly, developmental biology is highly interdisciplinary. Through conferences, our work has brought us in contact with diverse researchers from both developmental biology and parasitology, which has improved our perspectives and scholarship while being enormously fun to boot! Early on, while we were just getting up and running, Phil suggested we write an R21 grant application, not just for the practical matter of receiving funding, but to gauge if there was enough interest in our system, ideas, and methodologies, to merit support from our peers on study sections. Thankfully, the support of the NIH-NIAID was and continues to be encouraging and generous. Yet funding alone will not determine the success of this model. The real test will be whether students and post-docs choose to push this model forward, accelerate the research, and make their own careers with *H. diminuta* as at least one of their models of choice. I hope very much that my new lab at the University of Georgia will serve as an incubator for the success of this model and for researchers who will move it to heights I have not yet imagined!

H. diminuta, though impressively large in size, is still a model in its infancy. On top of the potential for translational relevance, I am excited by the promise of what we stand to learn. The limited nature of *H. diminuta* regeneration could allow us to uncover mechanisms that both restrict and enhance regenerative ability. The potency of tapeworm stem cell

subpopulations and their relationship to the microenvironment of the neck is still mysterious. Is there a pluripotent subpopulation or are multiple subpopulations able to regain/retain/maintain pluripotency as long as they receive the appropriate niche signals? What is the nature of the stem cell niche? What are the microenvironmental signals that regulate regeneration? How are segments initiated during regeneration and are they regulated by a segmentation clock? When are germ cells first formed and how are they coordinated with segmentation? These and many other questions are up for grabs. If you, dear reader, feel like wrangling tapeworms and unearthing their secrets holds a strangely irresistible (if somewhat queasy) appeal, the Rozario lab is looking for students and postdocs!



5. Concluding thoughts

This special issue exists because there is growing recognition that we need to complement the enormous advances we have gained from traditional model organisms with new emerging models that capture the breadth and diversity of the tree of life. Failure to do so would limit the scope of our knowledge and blind us to unique and extreme traits that already exist all around us (Goldstein & King, 2016). Realistically, we live in a world of limited resources so anyone attempting to establish a new/neglected model will be faced with this ultimate question—is it worth it? To us, we must choose our models because they are particularly well or uniquely suited to answer interesting scientific questions. With that criterion satisfied, the hard work—and it is hard work—will pave the way for an adventurous and rewarding ride!

Acknowledgments

We would like to acknowledge the many researchers not mentioned in this chapter whose excellent work could not be discussed in detail. We recognize that our success would not be possible without their contributions. None of this work would have been possible without the generous and flexible support of the Howard Hughes Medical Institute, of which PAN is an investigator. We are grateful to Melanie Issigonis for invaluable feedback in the writing of this manuscript. The figures were prepared by Matt Stefely at the Morgridge Institute for Research. Sincere thanks goes out to many colleagues and lab members who contributed significantly to the development of these models; specifically, the schistosome work was greatly aided by Fred Lewis, David Williams, Harini Iyer, Marla Tharp, Ryan King, Bo Wang, and Bram Lambrus, and the tapeworm work by Dick Davis, Jianbin Wang, Jennifer McDonald, and Edward Quinn.

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