Infective Larvae Production and Development in *Strongyloides* and its role in the dauer hypothesis

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
Alexander Dulovic
aus Kingston upon-Thames, Vereinigtes Königreich

Tübingen
2019

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	06.12.2019
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	PD. Dr. Adrian Streit
2. Berichterstatter:	Prof. Dr. Gerd Jürgens

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to my supervisor Dr. Adrian Streit, for his continuous support in allowing me to pursue my own research interests within his lab, and for his continued help, advice and guidance for the duration of my PhD. I would also like to thank Prof. Dr. Ralf Sommer for supporting me and allowing me to work in his department.

I am also very grateful to Prof. Dr. Gerd Jürgens for agreeing to evaluate this.

I would like to thank the current and former Parasitic Nematode group members, Anja Holz, Siyu Zhou, Tegegn Jaleta, Arpita Kulkarni, Mat Norman and Dorothee Harbecke, for their help in trying to understand more about this (at times irritating) group of parasites.

While I would obviously like to thank all of the Department 4, in particular I would like to thank Metta Riebesell for her help with microscopy over the years and Dr. Jim Lightfoot for his help with my sanity.

I would like to thank Dr. Christian Feldhaus and Aurora Panzera from the Light Microscopy Facility, and Iris Koch, Dr. Katharina Hipp, Brigitte Sailer and Dr. Mattias Flötenmeyer from the Electron Microscopy Facility for their help with my projects.

To my friends and family, I owe you a great deal for having to put with me over the last few years. In particular I would like to thank Krabbie, Marco and Nami for helping me with my move to Germany in the first place and Horst and Gabriele for making me feel so welcome.

Without my the independence that my parents gave me from a young age, I would never have considered moving to Germany, and the continued love and support that they give me has helped me immeasurably throughout my life.

Finally and most importantly, I would like to thank my fiancé. Your guidance and enthusiasm cannot be understated and I would not have been able achieve what I have without you.

TABLE OF CONTENTS

SUMMARY ..... **VII**

ZUSAMMENFASSUNG ..... **VIII**

LIST OF PUBLICATIONS ..... **IX**

INTRODUCTION **1**

NEMATODES 1
Nematodes *1*
Parasitic Nematodes *3*
Human Parasitic Nematodes *4*
STRONGYLOIDES 5
Strongyloides *5*
Life Cycle of Strongyloides *6*
Morphology of Strongyloides *9*
Sex Determination *10*
The Strongyloides Toolkit *13*
RNAI IN NEMATODES 14
RNAi in Nematodes – history and recent developments *14*
RNAi Mechanism *14*
SPERMATOGENESIS WITHIN NEMATODES 17
The Nematode Gonad *17*
Male Reproductive System in C. elegans *17*
Spermatogenesis in C. elegans *18*
Proteins involved in Spermatogenesis *20*
Strongyloides gonads and germline *21*
DAUERS, INFECTIVE LARVAE AND THE DAUER HYPOTHESIS 22
Dauers *22*
Infective Larvae *24*
The Dauer Hypothesis *25*
Host Sensation *25*
Genes and Pathways involved in Dauer Development *26*
The role of daf-12 and dafachronic acid in dauer and infective larvae development *28*
AIMS OF THIS THESIS 30

RESULTS AND DISCUSSION **33**

SYNOPSIS
Optimizing Culture conditions for the free-living stages of the nematode parasite Strongyloides ratti *33*
RNAi-mediated knockdown of daf.12 in the model parasitic nematode Strongyloides ratti *34*
Rhabditophanes diutinus, a Clade IV Parthenogenetic Nematode that makes dauer larvae *36*
UNPUBLISHED RESULTS 38
Strongyloides eliminates male-determining sperm through spermatogenesis *38*

UNPUBLISHED MANUSCRIPT 51
Rhabditophanes diutinus, a clade IV Parthenogenetic Nematode that makes dauer larvae 51

REFERENCES..... 97

PUBLICATIONS 124

SUMMARY

The dauer hypothesis for the evolution of parasitism in nematodes, states that dauer larvae, a specialised third larval stage present in many free living nematodes, served as a pre adaptation to parasitism and evolved into infective larvae (L3i). Unique biological features like alternating free-living sexual and parasitic parthenogenetic generations make *Strongyloides* spp., which includes the human pathogen *S. stercoralis*, an attractive model to study parasitism and the evolution thereof. One of the most striking features is that all L3is are female, although they can be produced from sexual reproduction. The main aims of this thesis were to test the dauer hypothesis for the specific example of Strongyloididae and to understand how female only progeny are formed from sexual reproduction.

While *Strongyloides ratti* is an attractive model species as it can be maintained within its natural host, its free living stages can only be extracted from fecal cultures and many tools that are standard in model organisms are not yet available, making experimentation difficult. Therefore, I firstly set out to increase the toolkit within *S. ratti*. I strongly improved the protocol to culture *S. ratti* on bacteria seeded agar plates, which led to highly increased fecundity and survival, allowing easier experimentation. Further I devised the first working procedures for RNAi in any species of *Strongyloides*. As *S. ratti* appears refractive to microinjection, I developed an RNAi by soaking protocol which works across multiple life stages and has minimal off-target effects. Using the aforementioned RNAi technique, I studied *daf-12* natively within *S. ratti* (the first study of its kind within *Strongyloides*). By suppressing DAF-12 I found that many functions of this nuclear hormone receptor are conserved between *C. elegans* and *S. ratti*. In particular, *daf-12(RNAi)* inhibited the formation of infective larvae as it does for dauer larvae in *C. elegans.* This demonstrates that the molecular mechanisms underlying dauer formation and L3i formation are conserved between species as phylogenetically distant as *C. elegans* and *S. ratti* , suggesting that DAF-12 it is a promising target for novel antihelminthics and providing support for the dauer hypothesis. In order to study the transition from free-living to parasitic, we need well-studied closely related free-living species to parasites of interest, of which we are currently lacking. After noticing that *Rhabditophanes KR3021* (the closest known free living species to *Strongyloides*) produces dauer larvae and arrested J2 larvae (both previously unreported) under starvation conditions, I aimed to characterise this species and provide clearly lacking basic information. In addition to providing a detailed description of life cycle and morphology, I showed that the *daf-12* pathway is also involved in dauer (but not arrested J2) formation making *Rhabditophanes / Strongyloides* a highly attractive system for the further evaluation of the dauer hypothesis. Next, by characterising spermatogenesis in *S. ratti*, *S. papillosus* and *P. trichosuri* (which does make males), I found that there is an uneven division resulting in viable X-bearing and non-viable nullo-X sperm. I showed that spermatogenesis itself is highly conserved within the Strongyloididae, from the presence of giant nuclei in the distal gonad, to expression and production of major sperm protein.

Zusammenfassung

Die Dauer Hypothese besagt bezüglich der Evolution von Parasitismus in Nematoden, dass Dauer Larven, ein spezialisiertes drittes Larvenstadium, das bei vielen freilebenden Nematoden vorkommt, als Präadaptation für den Parasitismus dienten und sich diese zu infektiösen Larven (L3is) entwickelten. Einzigartige biologische Merkmale wie die Abwechslung zwischen freilebenden sexuellen und parasitären parthenogetischen Generationen machen *Strongyloides* spp., zu denen auch das menschliche Pathogen *S. stercoralis* gehört, zu einem attraktiven Studienobjekt für Parasitismus und dessen Entstehung. Eines der auffälligsten Merkmale ist, dass alle L3is weiblich sind, obwohl sie durch sexuelle Reproduktion erzeugt werden können. Das Hauptziel dieser Doktorarbeit war es, die Dauer Hypothese für den spezifischen Fall der Strongyloididae zu überprüfen und zu verstehen, warum nur weibliche Nachkommen durch sexuelle Reproduktion entstehen. *Strongyloides ratti* stellt eine attraktive Studienspezies dar, da sie in ihrem natürlichen Wirt gezüchtet werden kann. Allerdings müssen die freilebenden Stadien aus Fäkalkulturen isoliert werden und viele Methoden, die bei Modellorganismen benutzt werden, sind noch nicht etabliert. Deswegen machte ich es mir zunächst zur Aufgabe, das methodische Repertoire für *S. ratti* zu vergrößern. Ich verbesserte das Protokoll zur Kultivierung von *S. ratti* auf mit Bakterien bewachsenen Agarplatten, was zu einer starken Verbesserung von Fruchtbarkeit und Überlebensrate führte. Des Weiteren entwickelte ich das erste funktionierende Protokoll für RNAi bei *Strongyloides*. Da *S. ratti* Mikroinjektionen schlecht zu tolerieren scheint, entwickelte ich ein Vorgehen durch Inkubation mit kurzen RNAs, das über mehrere Lebensstadien hinweg wirkt und nur minimale Nebeneffekte aufweist. Mit dieser Methode untersuchte ich *daf-12* bei *S. ratti* (die erste erfolgreiche Untersuchung dieser Art bei *Strongyloides*). Ich fand heraus, dass viele Funktionen dieses nukleären Hormonrezeptors zwischen *C. elegans* und *S. ratti* konserviert sind. Insbesondere verhinderte *daf-12(RNAi)* die Bildung von infektiösen Larven, genau wie es dies bei Dauer Larven von *C. elegans* tut. Dies zeigt, dass die molekularen Mechanismen, die die Bildung von Dauer/ L3i kontrollieren, zwischen phylogenetisch so weit auseinander liegenden Nematoden wie *C. elegans* und *S. ratti* konserviert sind. Dies legt nahe, dass DAF-12 ein vielversprechendes Ziel für neuartige Antihelminthika ist und unterstützt die Dauer Hypothese. Um den Übergang von freilebend zu parasitär untersuchen zu können, brauchen wir gut untersuchte, nahe mit den zu untersuchenden Partasiten verwandte, freilebende Spezies, von denen wir momentan nicht genug haben. Nachdem mir aufgefallen war, dass die mit *Strongyloides* am nächsten verwandte bekannte freilebende Spezies, *Rhabditophanes KR3021,* unter Laborbedingungen vorher nicht beschriebene Dauer und "arrested J2 Larven" bilden kann, beschloss ich, diese Spezies näher zu charakterisieren. Zusätzlich zu einer detaillierten Beschreibung des Lebenszyklus und der Morphologie zeigte ich, dass *daf-12* auch in dieser Art an der Dauer (aber nicht arrested J2) Bildung beteiligt ist, was *Rhabditophanes / Strongyloides* zu einem hochattraktiven System für die zukünftige Untersuchung der Dauer Hypothese macht. Schlussendlich charakterisierte ich die Spermatogenese bei *S. ratti*, *S. papillosus* und *P. trichosuri*. Der Prozess ist innerhalb der Strongyloididae konserviert, unterscheidet sich aber deutlich von *C. elegans*. Die beiden *Strongyloides* Arten, die keine männlichen Nachkommen erzeugen, unterscheiden sich aber von *P. trichosuri* (mit männlichen Nachkommen) dadurch, dass es während der Meiose eine asymmetrische Teilung gibt, die zur Bildung von überlebensfähigen Spermien mit und nicht lebensfähigen ohne X Chromosom führt.

LIST OF PUBLICATIONS

Parts of this thesis have been previously published elsewhere.

Optimizing culture conditions for the free-living stages of the model nematode parasite *Strongyloides ratti*

Alex Dulovic, Vadim Puller and Adrian Streit

Experimental Parasitology 2016. Volume 168, September 2016, Pages 25-30.
https://doi.org/10.1016/j.exppara.2016.06.005

RNAi-mediated knockdown of *daf-12* in the model parasitic nematode *Strongyloides ratti*

Alex Dulovic and Adrian Streit

Plos Pathogens 2019.. 2019 Mar; 15(3): e1007705.
Published online 2019 Mar 29. doi: 10.1371/journal.ppat.1007705

INTRODUCTION

Nematodes

- Nematodes

The phylum nematoda is broad and genetically diverse, with its constituents inhabiting a wide range of environments. Nematodes have successfully adapted to all environments found on earth, from all terrestrial niches through to marine and fresh water habitats [1, 2], including on and within plants and animals [3]. Recent estimates suggest that there around 25000 nematode species currently described [4], although there may be over a million in existence [5]. Nematodes themselves exhibit a remarkably diverse range of features, such as multiple modes of reproduction [6] or substantial differences in size and body plan. This ability to survive and thrive in multiple varied ecological niches in addition to having a wide variety of intriguing biological features (such as the variation in reproductive mode), makes them a highly interesting phylum for study, as evidenced by the fact that a nematode, *Caenorhabditis elegans,* was the first multicellular organism to have its genome sequenced [7].

Nematodes also exhibit a wide variety of different life cycles. One common feature to all nematode life cycles is the development through four moults from embryos through to adult worms (L1 to L4). Some nematodes also exhibit dauer stages [8] which allow them to survive extreme environmental conditions, whilst parasites often have infective larvae (both dauers and infective larvae will be further discussed later in the introduction).

The molecular phylogeny of the phylum nematoda has been studied for over 20 years, with a stable phylogeny consisting of five main clades now well established [9, 10], based upon the sequencing of the small subunit ribosomal DNA (*SSU*) [11] (Figure 1). As seen in Figure 1, parasites of invertebrates and vertebrates can be found across all clades whereas plant parasitic nematodes can be found in clades I, II and IV.

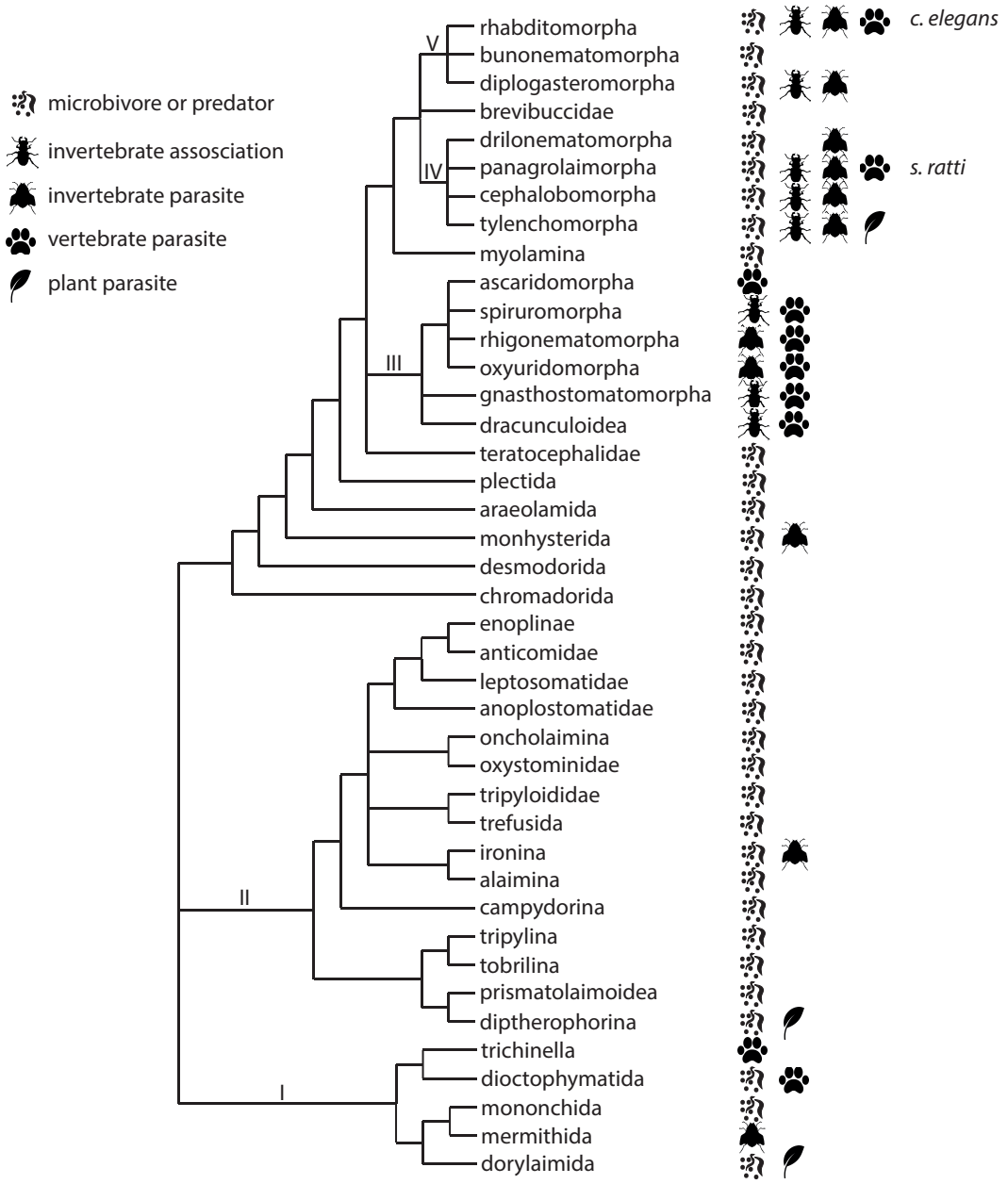


Figure 1: **Phylogeny of the phylum nematoda**. Adapted from [10, 11]. Dendrogram summarizing the phylogeny as determined by *SSU* sequencing. The 5 major clades are indicated by roman numerals (I – V), with common features of the taxa indicated by the symbols. Species of interest for the purpose of this thesis are indicated. It should be noted that all *Strongyloides, Parastrongyloides* and *Rhabditophanes* species discussed in this thesis are represented here by *S. ratti*.

- **Parasitic Nematodes**

Parasitism among nematodes is ubiquitous, in that almost all species of animals and plants are parasitized by nematodes. This has profound impacts upon agricultural yields (of both plants and livestock) and human health [2]. Phylogenetic analysis among animals indicates that parasitism has arisen over 200 times among 15 phyla [12], with at least 18 independent occasions within nematodes [10, 11]. This suggests that parasitism may in fact be the most natural lifestyle of all [13], yet it remains unclear why nematodes appear to have evolved into parasites so frequently compared to other phyla that have seen parasitism arise less often, or never in the case of some invertebrates such as echinoderma [12, 14]. This could be due to the fact that nematodes are so abundant and species rich that they simply have many more opportunities for parasitism to arise compared with other phyla [14]. Alternatively, it may be that becoming a parasite requires only a few evolutionary steps, something which can be easily achieved within nematodes due to their short generation time and comparatively small genomes [14].

Another potential explanation for this abundance of parasitism within nematodes is that nematodes frequently live in close association with other species. This degree of interconnectedness can lead to the nematode exploiting its niche environment and eventually becoming dependent upon the other species for its survival [14]. This degree of interconnectedness is not restricted to just parasitic nematodes, as the study of two free living model nematodes (*C. elegans) and Pristionchus pacificus (P. pacificus)*) shows that even free living worms can have strong associations with other species [15, 16]. Once outside of its host, the survival of a parasitic nematode depends upon its ability to withstand the adverse environmental conditions it finds itself in. Parasitic life cycles can be varied, involving single or multiple host species, with a large degree of variation in the exact life stage at which parasitic nematodes infect a new host [2]. For instance, *Ascaris* species survive outside the host and are directly transmitted to a new host whilst still within the egg shell [17], whereas

Strongyloides species survive outside of the host as filariform larvae known as infective larvae, the third stage of which are then able to infect a new host [18].

As the number of sequenced parasite genomes has increased [10], a number of insights into the genomic basis of parasitism have been gained. Although it was originally thought that parasite genomes were smaller than their free-living relatives, sequencing has shown that there is actually no real difference between the two, and that when such a difference does occur, it is normally a result of smaller introns and intragenic regions [10, 19]. Genomic analysis has also revealed extensive horizontal gene transfer between parasitic nematodes and bacteria/fungi, allowing the worms to adapt and parasitize new species. This is evidenced by the presence of cellulolytic enzyme genes in plant parasitic nematodes [10]. Meanwhile, analysis of genomes within the *Strongyloididae* family of parasitic nematodes was able to reveal gene families correlated with the expansion of parasitism [19].

Further knowledge about how free-living nematodes become parasites is desperately needed. With this knowledge we can begin to understand how parasitic nematodes find and infect their hosts, and later avoid the host´s immune system. Understanding these fundamental questions is not only of great importance for basic research, but also of great interest for medical, industrial and agricultural applications.

- **Human Parasitic Nematodes**

Human parasitic nematodes currently represent a great burden both economically and medically. Unsurprisingly, poor endemic regions are disproportionately affected by these parasites. Of the 20 most neglected tropical diseases (NTDs) listed by the World Health Organization (WHO), four are caused by parasitic nematodes [20]: *Dracunculus medinensis, Lymphatic filaraisis, Onchocerciasis* and *Soil transmitted helminthiasis (STH).* While guidelines and protocols have been established and developed to eradicate these diseases [21], eradication is rarely achieved and the development of novel treatments for these nematodes remains important.

Current antihelminthics used to treat parasitic nematodes are the Benzimidazoles (albendazole and mebendazole), Ivermectin and Levamisole [22, 23]. Current eradication programs, involve the mass deworming of school children across endemic regions using one or a combination of the above drugs [21]. However, whilst these drugs work effectively, there is the fear that resistance may arise as has already been reported within veterinary medicine [24]. This is particularly concerning for human health as reduced effectiveness has already been reported within *Onchocerca volvulus (O. volvulus)* and other filarial parasitic nematodes suggesting that resistance may already be evolving within human parasites [25-27].

Research upon human parasitic nematodes is often troublesome due to the complex life cycle and requirements of these parasites. This often means that suboptimal hosts or alternative closely related parasite species must be used instead in order to study the life cycle or biology. The multiple life stages and range of hosts required can also make maintenance of important parasites in a laboratory setting costly, both economically, ethically and in terms the physical space required.

Strongyloides

- ***Strongyloides***

Strongyloides are a genus of parasitic nematodes, with currently over 50 described species. They are known to infect mammals, birds, reptiles and amphibians [18], often being found whenever they are looked for. *Strongyloides* are often highly host specific, with most species able to infect only one or at most a few different species. Surprisingly, the different potential hosts are often not closely related. Notable examples of this are *Strongyloides papillosus (S. papillosus)* which is a natural parasite of sheep and is capable of also infecting cattle as well as rabbits [28, 29], and *Strongyloides stercoralis* which can infect both humans and dogs [30]. *Strongyloides* is a relatively common wild parasite, for example the prevalence of *Strongyloides ratti (S. ratti)*, the rat parasite, in wild rats in the UK is estimated to be around 62% [31].

In this thesis, I am presenting work on the rat parasite *Strongyloides ratti*, sheep parasite *Strongyloides papillosus*, the closely related Australian Brushtail Possum parasite *Parastrongyloides trichosuri* and the closely related free living species *Rhabditophanes sp. KR3021*. The phylogeny of these species can be seen in Figure 2.

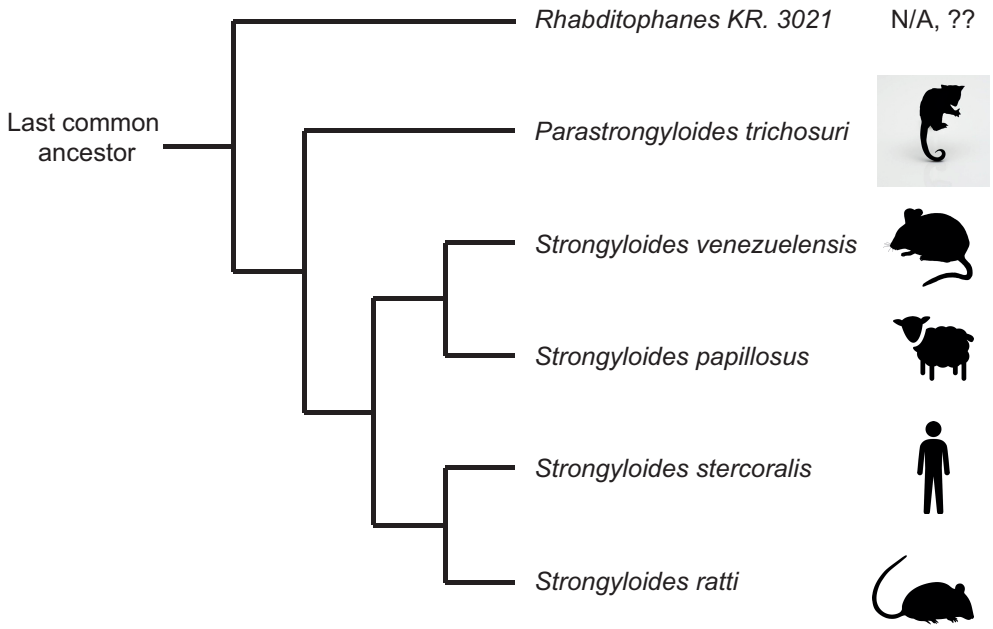


Figure 2: **Phylogeny of *Strongyloididae* and closely related species of interest**. Adapted from [19]. Tree branch lengths are not indicative of evolutionary history. Natural hosts for each species are indicated by the symbol, *Rhabditophanes* – unknown, *P. trichosuri* – Australian brushtail possum, *S. venezuelensis* – mouse, *S. papillosus* – sheep, *S. stercoralis* – human and *S. ratti* – rat. If *Rhabditophanes* should be included in the family Strongyloididae or only in the superfamily Strongyloididae is contentious.

- **Life Cycle of *Strongyloides***

The life cycle of *Strongyloides* spp. is one of the most fascinating aspects of the genus, featuring both internal host-dwelling and external environmental stages.

The life cycle of *S. ratti* as an example of the *Strongyloides* life cycle can be seen below in Figure 3.

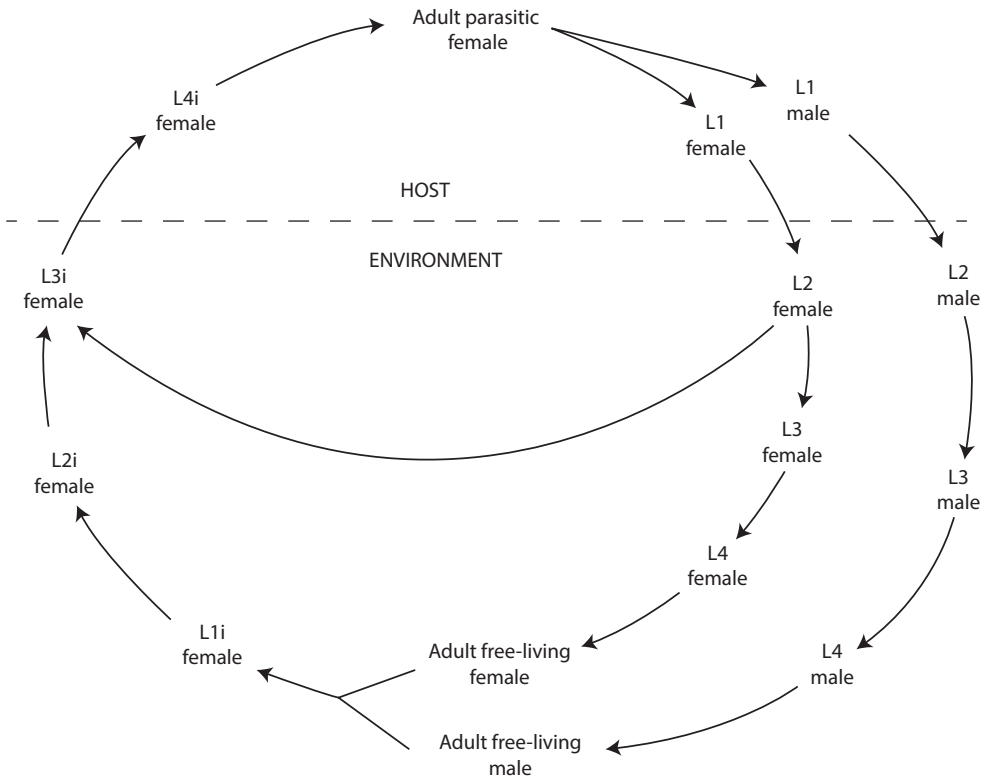


Figure 3: **Life Cycle of *Strongyloides ratti***. Adapted from [18]. The life cycle of *Strongyloides ratti* acts as a representation for the life cycle of *Strongyloides* species. It should be noted that the autoinfective cycle present in *S. stercoralis* and the reversion to L3 larvae from L2i larvae present in *P. trichosuri* are not shown here.

As seen in Figure 3, new hosts are infected by infective third stage larvae (L3i) which penetrate the skin [18]. These larvae then migrate through the body until they reach the lungs and nasal passages, where they are coughed up [32]. In a laboratory setting, this is normally achieved within 24 hours following a subcutaneous injection of infective larvae [32]. Once in the nasal region of the host, they are swallowed where they can reach the small intestine. During this migration to the small intestine, they undergo a moult to a L4i stage [33]. The

parasitic females then lodge into the gut mucosa, where they begin to reproduce and lay embryos [18].

The embryos released by the parasitic female hatch to a first larval stage (L1) while still in the host, and are then excreted from the host in the feces [18, 33]. The exact stage that they exit the host is variable among *Strongyloides* species, with some exiting while still as embryos [28, 34], while others exit as already hatched larvae [18]. The L1 then moult to become second stage larvae (L2) in the environment. From here, the females have a decision towards undergoing either direct development and moulting directly to L3i and infecting a new host, or undergoing indirect development and completing moults from L2 (via L3 and L4) to free living adults. Males can only undergo indirect development via moulting from L2 to become free-living adults (also via L3 and L4). These free living adults then reproduce sexually resulting in a female only generation [18]. These females then progress via moults from infective first stage larvae (L1i) to infective third stage larvae (L3i).

Most *Strongyloides* species follow the above life cycle of having alternating free-living and host-dwelling cycles, however there are some notable exceptions. The human parasite *Strongyloides stercoralis* is capable of undergoing an autoinfective life cycle. This happens when L1 larvae transform from rhabditiform larvae to filariform infective larvae and penetrate out of the intestinal wall or perianal skin, migrating and reinfecting the same host, resulting in a successive generation being formed within the host [35, 36]. This allows an infection to persist indefinitely within the host [37] and is fatal [35]. Within the cat parasite *Strongyloides planiceps*, multiple free-living generations (up to nine) are possible as opposed to the single free living generation within every other species studied [38].

For the closely related parasitic species *Parastrongyloides trichosuri*, indefinite free-living generations are possible [39]. Otherwise *Parastrongyloides* follows the exact same life cycle and apparent migration pattern as in *Strongyloides*, with the exceptions that L2i larvae may also revert back to being L3 larvae instead of

developing to L3i larvae, and that offspring of both sexes are produced following the adult sexual reproduction event resulting in the gonochoristic sexually reproducing parasitic generation present in this species [39-41].

The closely related free-living species *Rhabditophanes sp. KR3021*, follows a similar life cycle to other free-living species such as *C. elegans*. The adults lay embryos that mature through a series of four molts from L1 to become adults again [19]. Under difficult environmental conditions, this species is capable of generating low levels of dauer larvae and arresting J2 larvae to allow it to survive for an increased length of time (part of this thesis). It is currently unknown if *Rhabditophanes* has a natural host species [42].

- **Morphology of *Strongyloides***

For all species of *Strongyloides* and *Parastrongyloides*, the parasitic females are found within small intestine of infected hosts [18, 40]. They are around 2 to 5 mm in length, with an elongated and straight-sided oesophagus and a blunt ended tail [29, 43]. The ovary is didelphic, leading to the vulva which can be found around two-thirds of the way down the body [43]. An example of parasitic females can be seen in Figure 4A.

The free-living adults are more variable in size, although most are around 1mm in length [18]. Unlike the parasitic females, they have a rhabditiform oesophagus. The free-living females have a didelphic ovary which leads to a vulva, found at a midpoint of the body [43]. Developing embryos can be seen along the length of the gonad arm once the individual is mature enough. An example of a free-living female can be seen in Figure 4B. Free-living males have a single gonad stretching the length of their body, with sperm accumulating towards the distal end, with a single spicule found above the anus [18]. An example of a free-living male can be seen in Figure 4C.

Infective larvae which are produced through indirect development (by sexual reproduction) undergo two molts from L1i larvae to L3i larvae. While infective larvae that are produced by direct development also have two molts, they develop directly from L2 to L3i. The L1i are indistinguishable from L1 as they

also have a rhabditiform oesophagus. L2i also have a rhabditiform oesophagus, however the L3i larvae not only have a filariform oesophagus, but they also a longer thinner body form [43]. L3i larvae can be seen in Figure 4D.

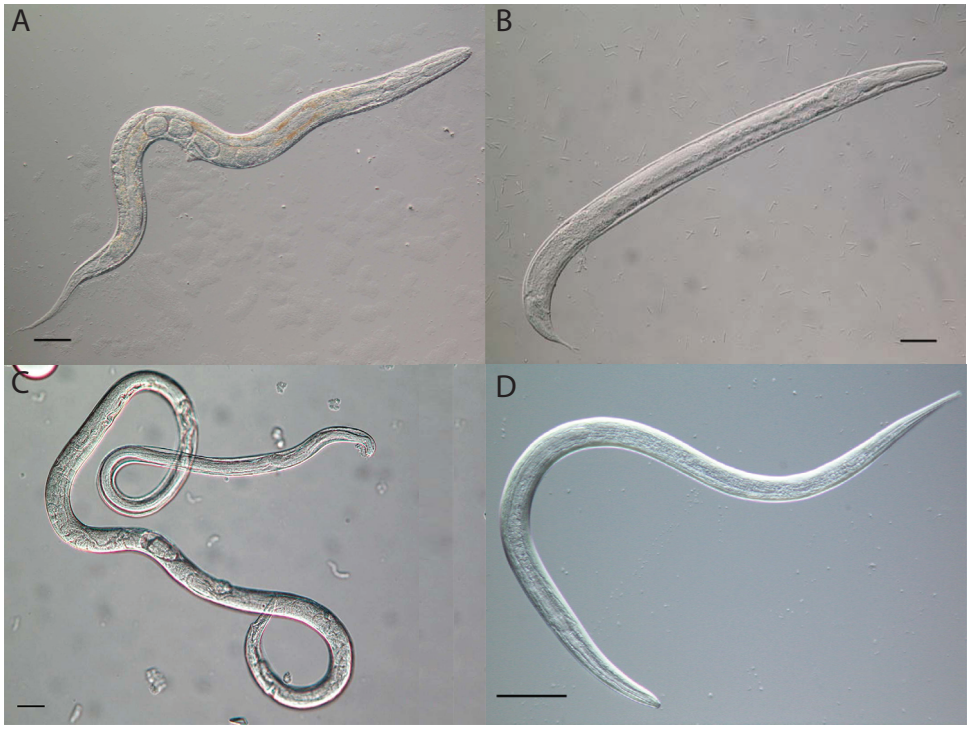


Figure 4: **Images of different lifestages of *Strongyloides ratti***. Reproduced from [18]. Adult females (A) and adult males (B) are substantially smaller than Parasitic Females (C) or Infective Larvae (D). Scale bars are 50µm in A and B, 30µm in C and 100µm in D.

- **Sex Determination**

Organisms have evolved various modes of reproduction, from gonochoristic species with separate sexes to hermaphroditic and parthenogenetic species that reproduce from a single parent [44]. Between these extremes, mixed reproductive systems may also occur, from gynodioecy (with females and hermaphrodites), to androdioecy (males and hermaphrodites) and trioecy (females, males and hermaphrodites) [45]. Among most parasitic species, gonochoristic individuals (both male and female) exist, with sexual reproduction occurring between the two. The gender of their offspring however, can be determined through genetic sex determination (GSD) or environmental sex determination (ESD), with both systems present within nematodes [6]. A genetic

system whereby females have two X (XX) chromosomes and males only one (X0), appears to be the most common and possibly the ancestral system within nematodes [6, 46].

Strongyloides appear to have made the transition from GSD to ESD relatively recently [47]. For all species of *Strongyloides* that have been studied in detail so far, the sex ratio of the offspring from the parasitic female within the host is affected by the hosts' immune status [47, 48]. An increase in immune response within the host results in an increase in male production [47, 49]. This environmental driven sex determination system has probably only recently evolved from the genetic XX/X0 system, as this is still present within the closely related species *P. trichosuri* [39, 41].

S. ratti has a XX/X0 system whereby female offspring have 2 copies of the X chromosome in addition to 2 pairs of autosome, whereas males only have a single copy of the X chromosome [50]. In *S. papillosus* however, only 2 pairs of chromosomes are present [34, 51]. The X chromosome and Chromosome I are fused together to generate a pair of large chromosomes, in comparison to the other autosome [19, 34]. In males, one copy of the X chromosome is removed by chromatin diminution, resulting in 1 large, 3 medium and 1 small chromosome [34, 51]. Chromosome numbers for *S. ratti* and *S. papillosus* can be seen in Figure 5.

As stated before, chromatin diminution is present within *S. papillosus.* Chromatin diminution is a developmental process whereby portions of the genome are reproducibly eliminated [52] and can be found across a wide range of eukaryotic species [53]. Within nematodes, chromatin diminution appears limited to two orders, the Ascardida (*i.e. Ascaris suum*) and the Rhabditida (*i.e. S. papillosus*) [52].

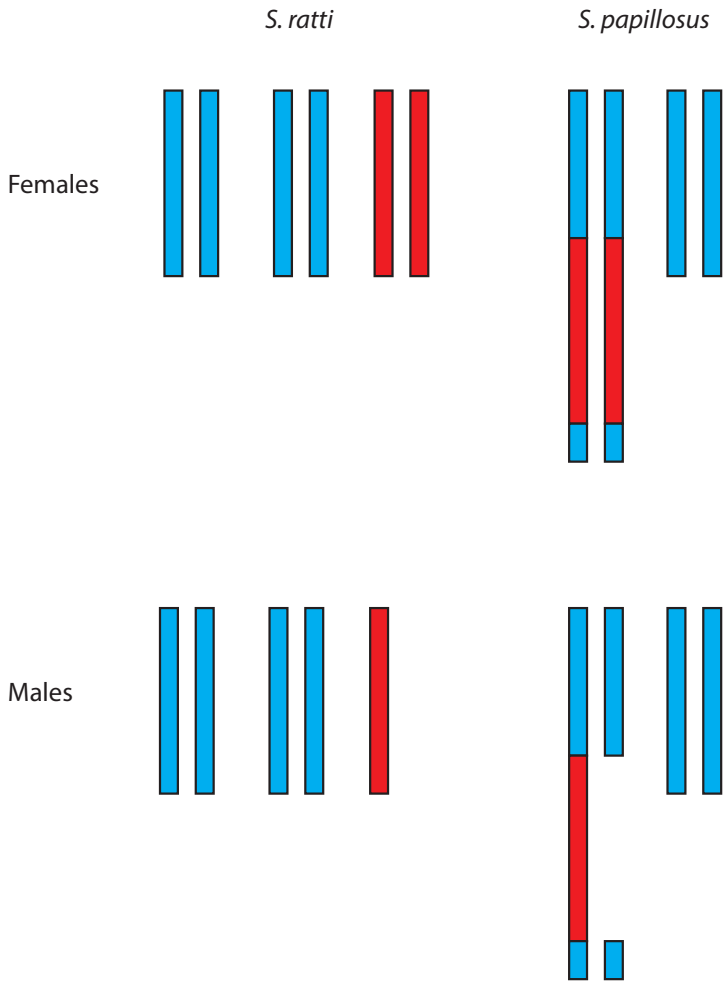


Figure 5: **Chromosome complements in *Strongyloides ratti* and *Strongyloides papillosus*.** Adapted from [47]. Chromosomes and parts of chromosomes present in two copies in both sexes (as functional autosomes) are shown in blue, while chromosomes and parts of chromosomes present in two copies in females (XX) but only one copy in males (X0) are shown in red. The XX/X0 sex determination system can be seen clearly, as can the male specific chromatin diminution event present in *S. papillosus*. *P. trichosuri* has the chromosome complement as *S. ratti.*

Chromatin diminution within *Strongyloides* differs to that present within Ascarids for several reasons:

- Firstly, it creates a difference in DNA on the basis of sex and not between the soma and germ line [52].

- Secondly, only a single copy of the genome is removed in *Strongyloides*, not both [52]
- Thirdly it does not appear to remove predominantly repetitive sequences as these are less common within *Strongyloididae* genomes [19, 52]
- Fourthly, it is induced by environmental cues in *Strongyloides* [52]
- And finally, it only occurs at the one cell stage in *Strongyloides* [34, 52]

These differences suggest that chromatin diminution has evolved separately within nematodes, as is similar in other metazoans [52, 53].

- **The *Strongyloides* Toolkit**

Early attempts at forward genetics within *Strongyloides* resulted in the establishment of EMS (Ethyl Methyl Sulfonate) to generate mutants [54]. *Strongyloides* and *Parastrongyloides* were then the first parasitic nematodes to undergo successful transformation, using plasmid based constructs [55-57]. As the body plan between *Strongyloididae* females and *C. elegans* hermaphrodites is similar, this allowed the implementation of gonadal microinjection protocols from *C. elegans* [58-61].

In order to establish a successful line of expressed transgenes in these parasites, the piggyBac transposon system [62] was co-opted to allow stable expression in successive generations of *S. stercoralis* [59, 63]. This system was later established within *S. ratti* [64]. While this allowed expression patterns of genes to be understood [65, 66], the ability to knockdown genes of interest to study their function was still not present.

With the advent of CRISPR/Cas9 technology [67], it has never been easier to mutate and study genes of interest within nematodes . While in model free-living organisms like *C. elegans* and *P. pacificus*, the technique was quickly established [68, 69], in *Strongyloides* it took far longer for the first successful mutagenesis [70]. However, while mutants of interest can now be easily generated, no lines of mutant offspring are currently in existence due to the large number of infective larvae required to start an infection in gerbils with *S. stercoralis*. As a result, the establishment of CRISPR/Cas9 in *S. ratti* was of high

importance as it is possible to generate single isofemale lines. Unfortunately, *S. ratti* appears rather refractive to microinjection and the incorporation of plasmids as seen by the lower mutation and survival rates of injected worms reported by multiple authors [64, 70].

To this end, the development of non-microinjection protocols such as RNAi by soaking ([71], part of this thesis) along with further methodological improvements of CRISPR within *Strongyloides* [72] are currently the focus of much attention.

RNAi in nematodes

- RNAi in nematodes – history and recent developments

RNA interference (RNAi) involves the inhibition of gene expression by RNA molecules [73]. The RNAi pathway is conserved among eukaryotes, and as a result RNAi has been established as a technique for the suppression of genes within many model organisms [74-77].

RNAi has long been used within the model nematode *C. elegans* [73, 78] and within plant parasitic nematodes like *Meloidogyne incognita* [79], *Heterodera glycine* [80] and *Globodera pallida* [80]. More recently, RNAi has also begun to be established within entomopathogenic nematodes like *Heterorhabditis bacteriophora* [81] and *Steinernema carpocapsae* [82] and even more recently within animal and human parasitic nematodes like *Haemonchus contortus* [83] and *Brugia malayi* [84]. While it had previously been thought that *Strongyloides* species were refractive towards RNAi [85], this was based upon the application of long dsRNA molecules. Recent developments have begun instead to use small interfering RNAs (siRNAs) or small hairpin RNAs (shRNAs) for RNAi within parasites [84, 86], suggesting that *Strongyloides* may nevertheless be susceptible to RNAi.

- RNAi mechanism

The overall process for RNAi can be seen in Figure 6. RNA (normally as exogenous dsRNA or siRNA) is imported into the cytoplasm of the cell from the

extracellular environment, or produced endogenously from within the nucleus. Following this, siRNA or miRNAs are generated from the original RNAi. To achieve this, the RNA is transported to the Dicer complex (a mixture of ribonuclease proteins), activating it, resulting in the binding and cleaving of RNA into shorter fragments [87, 88]. These shorter RNAs are then integrated into a RISC (RNA Induced Silencing Complex), a nuclease complex of proteins which binds these shorter RNAs [89]. Following binding, the antisense strand is kept allowing for mRNA target binding whereas the sense strand is ejected [87]. This antisense strand guides the RISC complex to the complementary target mRNA where it binds and cleaves it [90]. This prevents the mRNA from being used as a translation template which therefore prevents the gene from being expressed. It also possible for the RNAi signal to be amplified. This occurs when some of these siRNAs are amplified by RNA-dependent RNA Polymerases (RdRPs). This amplification can allow the RNAi signal to spread from cell to cell, silencing expression in the neighbouring cells, resulting systemic environmentally induced RNAi [91]. Alternatively, these siRNAs following RdRP amplification can enter the nucleus for further silencing [91]. The siRNAs can also be inhibited by intracellular negative regulators [91].

C. elegans appears to have a far higher amount of RNAi effectors compared to other species of nematodes [87]. It is unclear whether this is indeed the case or whether it is due to the sampling bias of *C. elegans*, which has led to more protein functions being elucidated, or whether nematodes have alternative proteins or pathways that have yet to be characterized.

Of these proteins, it was known that transmembrane protein SID-2 was required for dsRNA uptake from the environment in *C. elegans*, as this gene is lacking in the closely related species C*. briggsae* which is refractive to RNAi by soaking [92]. However, recent bioinformatic analysis of parasitic species of nematodes in which RNAi works, showed they also lack this gene and that this gene is not present outside of the *Caenorhabditis* genus, confirming that the genes required for RNAi are not conserved between all nematodes, and that alternative as yet undescribed proteins or mechanisms are present [87]. The differences in the

presence of RNA uptake proteins between nematodes could also be attributed to differences in the cuticle thickness, structure or permeability which may affect uptake and propagation of RNAs [87].

In C. elegans, plants and other species, RNAi is amplified by RNA dependent RNA polymerases (RdRPs), which produce a further population of siRNAs [93]. These RdRPs while conserved to a certain degree, are unique to each species [94].

The RISC complex consists of TSN-1, AIN-1, VIG-1 and AIN-2 [95, 96]. These proteins are some of the most conserved between nematodes, with a recent study of parasitic nematodes genomes finding that all tested species had at least one of the proteins, suggesting they are capable of forming a RISC complex [87]. This family of proteins is also heavily conserved within *Strongyloididae* with all tested species having all four proteins present, suggesting they can form RISC complexes ([71], part of this thesis).

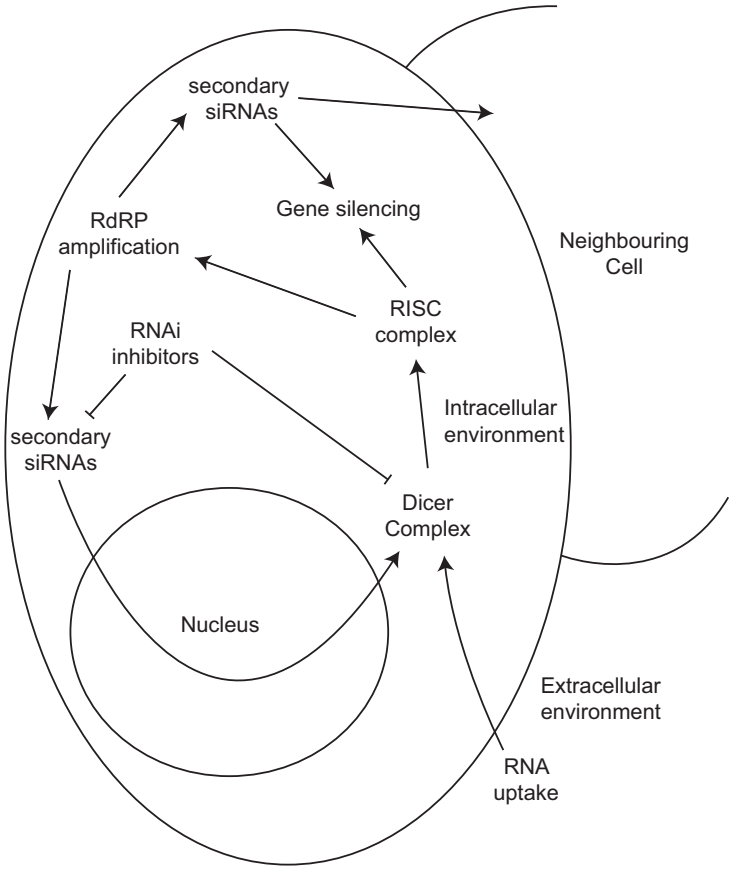


Figure 6: **Schematic representation of RNAi pathways present in *C. elegans***. Adapted from [87].

Spermatogenesis within nematodes

- The nematode gonad

The nematode gonad is highly variable [6, 97]. It comprises typically around 60% of the volume of the adult nematode, and can be several times the length of the nematode when removed and unfolded [98]. The gonad is involved in not only the production of gametes, but also the development of other organs, lifespan decisions and even pheromone production [99-101].

The variability of the gonad can be due to the number of gonad arms present. Some species have a single arm (monodelphy) whereas other species have two arms (didelphy) [102]. There is also variation in the position of the gonad within the body of the nematode, with some gonads located in the anterior of the body and others in the posterior [102]. The location of the vulva is also highly variable [103]. Most female or hermaphroditic gonads are didelphic, which may be either symmetrical or asymmetrical [102]. By contrast, male gonads are always a single arm with a spicule at the end, which allows the release of sperm by identifying the vulva and then holding it open during ejaculation [104, 105].

A key feature of the gonad is the germline. The germline consists of mitotic cells and meiotically dividing cells in different stages of development differentiated, resulting in the formation of gametes and allowing the transfer of genetic material to the next generation). In males, the germline generates only sperm and does so for the entire life of the animal [106], whereas in females it generates oocytes and in hermaphrodites, both oocytes and sperm. While females produce relatively low numbers of eggs (as they are large and require relatively extensive resources), males by comparison produce large amounts of sperm, which are usually small, highly motile, and prepared for fusion to produced a fertilized embryo [107].

- Male Reproductive system in *C. elegans*

The male reproductive system consists of all of the tissues involved in either gamete production or copulation: the somatic gonad and its relevant neurons,

the male germ line and the proctodeum and its associated muscles and neurons [108].

In *C. elegans*, the somatic gonad consists of the non-germ line components of the gonad arm [108], specifically the distal tip cells, seminal vesicle and vas deferens [109]. The *C. elegans* male gonad can be seen in Figure 7A. The distal tip cells are required to maintain a population of proliferating germ cells [109], while the seminal vesicle stores spermatids until ejaculation [110] and the vas deferens appears to regulate sperm release [108].

The male germline results in the production of male only gametes known as sperm. Gametogenesis occurs in the proximal germ line with spermatids then stored in the seminal vesicle until ejaculation [108, 111]. These sperm remain inactive until ejaculation [112]. In the distal portion of the gonad, germ cells in *C. elegans* have incomplete borders and are connected by a central rachis. The distal end of the male germline contains a population of stem-cell-like cells, which then undergo development through meiosis. Different stages of sperm cell formation can be seen easily with DAPI staining (Figure 7B).

- **Spermatogenesis in *C. elegans***

Spermatogenesis is the process that results in the creation of sperm from previously undifferentiated germ cells [113]. In *C. elegans*, both the hermaphrodite and male worms produce sperm [113]. While there are some differences between them, I will focus on the males as it pertains to this thesis.

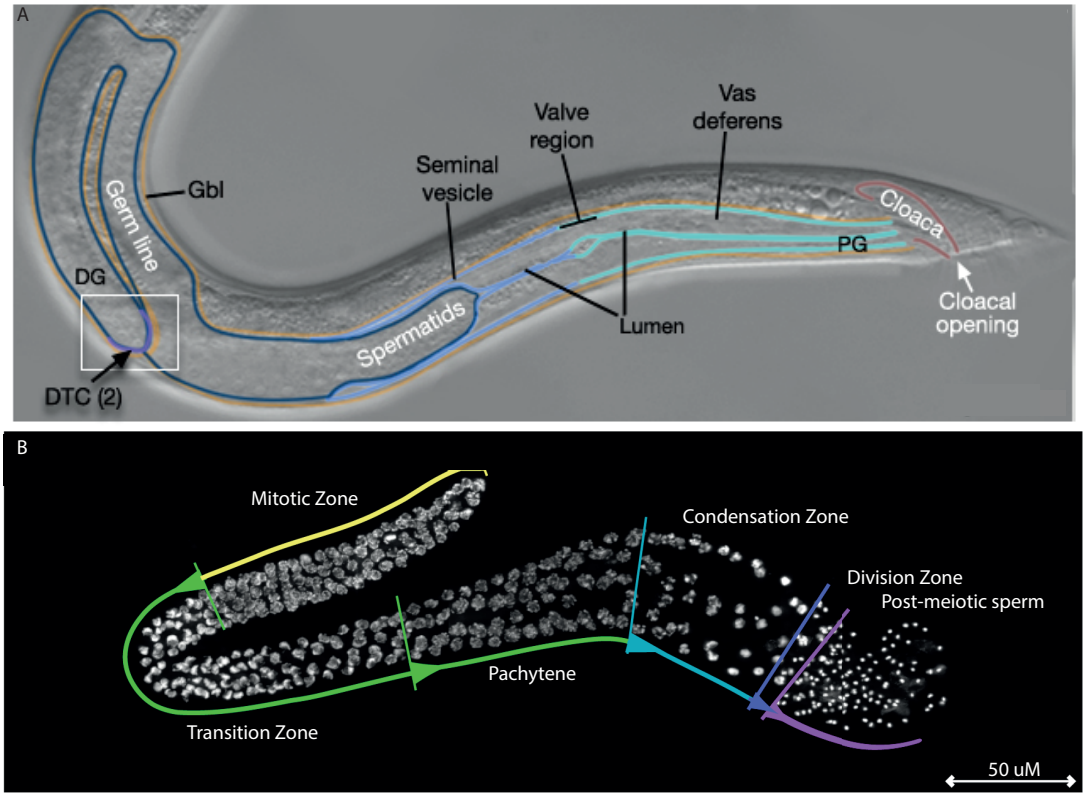


Figure 7: **The male gonad in *C. elegans* both under DIC and dissected out and DAPI stained to reveal the germline**. A is reproduced from [108] and B is reproduced from [114]. A shows the morphology of the male gonad in *C. elegans* including important anatomical structures in addition to the germline. DG – distal gonad, Gbl – Gonad basal lamina, PG – proximal gonad. B shows the stages of the germline from the mitotic zone through to sperm. Colours indicate the different zones of the gonad.

The overall process for spermatogenesis in *C. elegans* can be seen in Figure 8. Initially, the primary spermatocyte is produced in the syncytium with a cytoplasmic core-like structure called the rachis. Upon beginning spermatogenesis, the primary spermatocytes bud off the rachis and undergo the first meiotic division to form two secondary spermatocytes. These secondary spermatocytes immediately undergo the second meiotic division to form two spermatids. This means that 4 spermatids are produced from each primary spermatocytes. The spermatids are attached to a residual body where they develop before budding off. Spermatids than mature to spermatozoa under the control of external and internal proteins, resulting in the formation of a single

pseudopod. This process is not automatic and is controlled by extracellular signals. In *C. elegans* males, activation is concurrent with mating and ejaculation and is under the control of swm-1 and try-5 [115]. The sperm are then able to crawl using this pseudopod [113, 116].

Mature *C. elegans* sperm are stable, polarized cells. Their cells bodies contain a typical arrangement of organelles and chromatin [112]. Whilst chromatin and centrioles remain centrally located within the sperm, with mitochondria in an intermediate position, the periphery is partially invaginated as a result of membranous organelle [112]. These membranous organelles contain major sperm protein, responsible for the formation of the pseudopod [112].

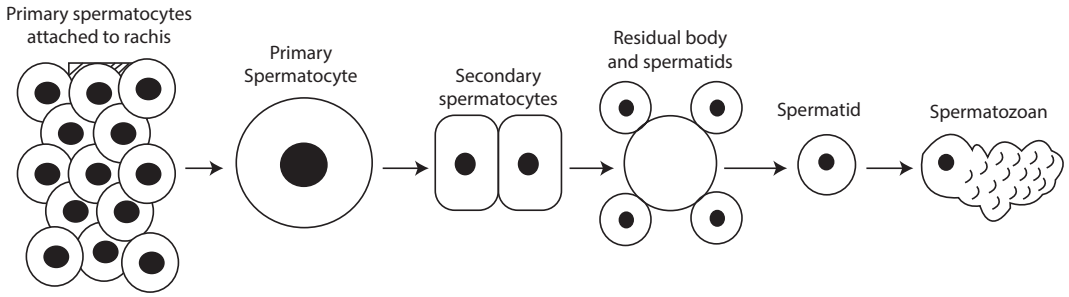


Figure 8: **Schematic pathway for spermatogenesis in *C. elegans***. Adapted from [108, 113].

- **Proteins involved in Spermatogenesis**

In *C. elegans*, the primary spermatocytes which are still attached to the rachis, have intact nuclear envelopes consisting of laminin and other nuclear pore complex proteins [114]. The division of spermatocytes is driven by tubulin, for which characteristic spindles can be seen when stained [114, 117]. Interestingly, the synaptonemal complex, a proteinous scaffold that assists with recombination, is degraded prior to karyosome formation, when the chromosomes are still fully condensed [114]. Sperm maturation itself is strongly linked to the expression of Major Sperm Protein (MSP). MSP is a small molecule, 14kDa in size, which eventually forms the cytoskeleton of the pseudopod [118-120]. During *C. elegans* spermatogenesis, MSP exhibits a strong localization pattern. Once synthesized, it initially forms fibrous bodies within developing spermatocytes, which are then segregated specifically into the

spermatids following the second meiotic division [117, 121]. Once the fibrous bodies have disassembled, MSP is distributed throughout the spermatid cytoplasm [117, 121]. Finally, when sperm activation is occurring, MSP is localized to the pseudopod where it functions as the major motility protein involved in the crawling process [117, 121]. In order to allow the sperm to deposit cellular components it no longer requires for spermatogenesis, the residual body functions as a trash can, removing proteins such as myosin [122] or MSP [117] from maturing spermatids.

- ***Strongyloides* gonads and germline**

While the overall morphology of *C. elegans* and *Strongyloididae* gonads is fairly similar, there is a large degree of differentiation particularly in the appearance and organization of the germline [123-125]. The overall organization of the *Strongyloides* gonad can be seen in Figure 9. As seen in Figure 9, the distal arm of the gonad in both sexes of *Strongyloides* consists of giant nuclei. These giant nuclei are DNA heavy, having a DNA content of upto several hundred C [125-127]. Following these giant nuclei is a band of small nuclei. In females, this small nuclei is located at the gonad loop (loop is straightened in Figure 9) whereas in males it is found approximately half way down the gonad. Following these small nuclei are the sperm or oocytes, depending on sex. In males, the actively dividing sperm are located just after the small nuclei with mature sperm located at the bottom (Figure 9).

Strongyloides lack mitotic cell divisions within the germlines, yet appear to have a much smaller distal tip like cell which does not cap the entire gonad tip [125]. It is currently unknown how exactly the gonad is formed in *Strongyloides*. It is thought that the giant nuclei may function as nurse cells [126, 127] providing a continuous supply of germ cells. Given that female only offspring result from a sexual reproduction event, it is also unknown how exactly this occurs. It is thought that XX sperm are preferentially made over X0 sperm or that X0 sperm have impeded development, as some X0 sperm does exist within *Strongyloides ratti* [127]. By contrast, *Strongyloides papillosus* completely lacks X0 sperm [127]. Currently however, nothing is known about the process of

spermatogenesis within *Strongyloides*, making it difficult to understand the lack of male offspring in this genus (the study of this forms part of this thesis)

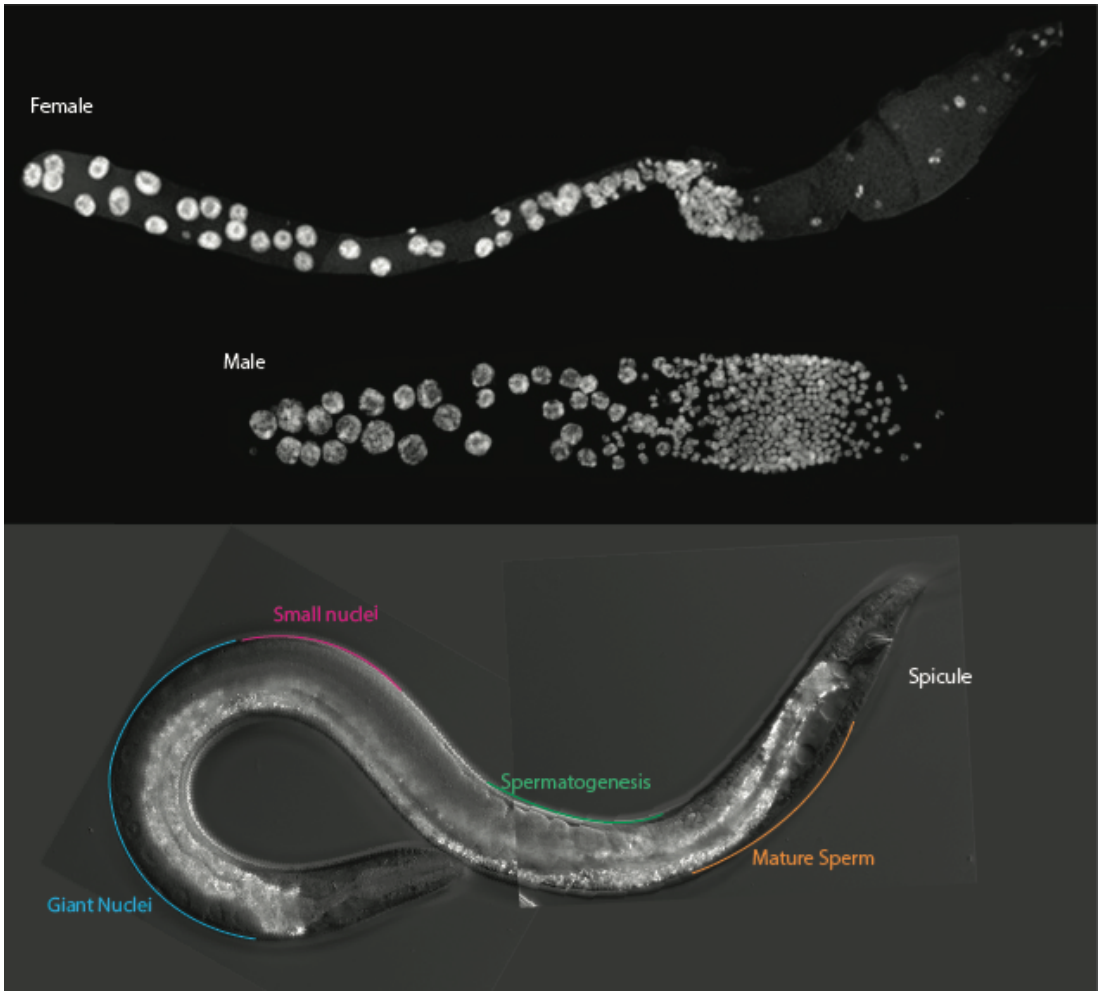


Figure 9: **Dissected Gonads of *Strongyloides ratti*** (reproduced from [125]) **and *S. ratti* male under DIC observation**. The male gonad has been annotated to show its different features and stages of spermatogenesis.

Dauers, Infective Larvae and the Dauer Hypothesis

- **Dauers**

In response to unfavourable environmental conditions, some nematode species such as *C. elegans* are able to form arrested larvae known as dauers. The dauer larvae are formed after the second molt by arresting their development [128]. This results in morphologically and behaviourally different larvae (compared to

those found in non-harsh environments), which have the ability to survive until the conditions have improved [8, 128]

The decision to become dauers occurs during the L1 larval stage and is dependent upon at least three factors: population density, food supply and temperature [8]. The relative amounts of pheromone present (indicating population density) and food signal are critical to determining whether dauer arrest occurs or not [129], while increasing temperature results in increased dauer arrest [130]. Recent work has uncovered the pheromones involved in dauer production in *C. elegans* [131], yet these are slightly different to those involved in dauer production in *P. pacificus* [132, 133].

Dauers themselves have clear morphological differences to normally developing larvae. Examples of dauer larvae of *C. elegans* can be seen in Figure 10. Dauer larvae are radially constricted, resulting in a longer, thinner worm, with a specialized cuticle [128]. They have a buccal plug [130], resulting in a closed mouth while their pharynxes are also highly constricted [134]. This buccal plug also prevents them from pumping, meaning they do not feed or take up any material from their environment [135]. Their germline is arrested, with shrinkage too of the muscle, intestine and hypodermal cells [136]. To survive, they metabolise their intestinal and hypodermal lipid stores [136]. Despite this, they are highly motile as they explore the environment while searching for food sources [128]. Overall they are highly long lived compared to other larval stages [137]. They exhibit behaviours such as nictation, enabling them to carried along by passing objects to new environments [136]. Once a food source is located, dauers resume normal development into adults, via an L4 molt. Dauers can be easily isolated by treatment with SDS [128]. Worms lacking a buccal plug are killed by the SDS, resulting in a homogenous dauer population. As a result, SDS treatment remains the gold standard for determining whether a worm is a dauer or not.

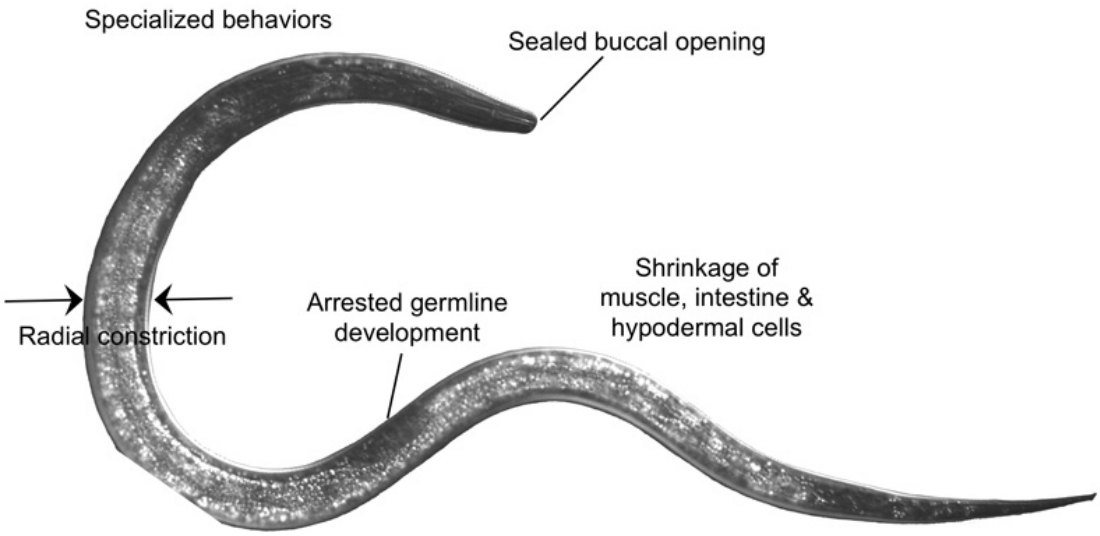


Figure 10: **Dauer Larva of C. elegans**. Reproduced from [136]. Typical features of the dauer larvae are shown.

- **Infective Larvae**

Parasitic nematodes feature a developmentally arrested infective larval stage that is responsible for making the transition to the host. These infective larvae bear a strong morphological similarity to dauer larvae of free-living nematodes [138]. Other infective larvae however are less morphologically similar to dauers.

Infective larvae often possess a modified cuticle, consisting of both the retained second stage cuticle and the new third stage cuticle, which resembles the dauer cuticle in ultrastructure (sheathed larvae) [138]. This second stage cuticle is then shed during the process of infection. *Strongyloides* species contain an non-sheathed third stage filariform infective larvae [18], in which the moult from L2i to L3i has occurred, and development has then been arrested. These L3i contain a specialized cuticle that is lost during the process of infection [139].

For obligate parasites such as *Haemonchus contortus*, the formation of infective larvae is constitutive. For Strongyloididae, development is often influenced by the host immune status or environment [48]. In *P. trichosuri*, formation of L3i is mediated by a constitutively secreted pheromone, similar to the dauer pheromones secreted by *C. elegans* [140]. In *Strongyloides,* infective larvae that are formed through direct development can be influenced by the same external

factors, however those that are formed through indirect development are formed external factors as part of the life cycle

Infective larvae possess many of the same phenotypic and biological characteristics as dauers. For example, both have a radially constricted body plan, exhibit nictation behavior, do not feed and are capable of prolonged survival in their arrested state [2]. These shared characteristics between dauers and infective larvae resulted in the dauer hypothesis

- **The Dauer Hypothesis**

The dauer hypothesis states that infective larvae evolved from a dauer larvae precursor and that similar mechanisms govern infective larvae and dauer development [141]. That dauer larvae exist and form necromenic or phoretic associations with other organisms appears to support this hypothesis further. For example, the free living nematode *P. pacificus*, features a necromenic association with beetles [16]. These nematodes remain as dauer larvae on a beetle, but develop into free living stages in the wild or in a laboratory setting until their food source is exhausted, at which point they develop into dauer larvae again [16].

- **Host Sensation**

One crucial aspect of infective larvae behavior is the ability to find a new host. As parasitic species are often highly species specific, they must be able to find and identify the right host before invasion. The mechanisms involved in this are currently unclear.

Recent work has begun to characterize the host seeking behaviors of parasitic nematodes [142]. *S. stercoralis* is known to exhibit positive chemotaxis towards molecules indicative of a new host [142]. They and other parasitic species like *Ancyclostoma caninum* (*A. caninum*) also exhibit positive chemotaxis towards CO_2 [142, 143], although this can be explained in the context of their life cycle. Infective larvae of both species migrate to the lungs, an area of high carbon dioxide concentration, to complete their molt to L4i whilst in the body. Urocanic

acid also acts as a strong positive chemoattractant of *S. stercoralis* infective larvae [144]. *A. caninum* infective larvae meanwhile are able to sense and respond to skin washes and temperature changes [145]. In order to better understand the nature of parasitism, further work understanding the mechanisms of host invasion and sensation must occur.

- **Genes and pathways involved in dauer development**

As previously mentioned, entry into and exit from dauers in *C. elegans* is controlled in response to temperature, population density and food availability [8]. These external environmental stimuli are detected by sensory neurons [146] and four molecular pathways: cyclic GMP, TGF-ß, Insulin and Steroid hormone signaling. An overview of the dauer signaling pathway can be seen in Figure 11.

As seen in Figure 11, the cyclic GMP Pathway is initiated when pheromones produced in response to environmental crowding, are sensed by GPCR receptors such as SRBC-64. This signal is then transduced by G-proteins GPA-2 and 3 to the guanylyl cyclase receptor DAF-11 and DAF-21. These then suppress DAF-7 and DAF-28 expression via the nucleotide gated channel subunits TAX-2 and TAX4 [141]. Of the genes involves in the cGMP pathway, TAX-2 and TAX-4 are heavily conserved genes, present in multiple parasitic species [147]. However, DAF-11 is limited to clades III to V, while other sensory proteins such as SRG-37 appear unique to C. elegans [147]. This suggests some divergence between the pathways controlling dauer and infective larvae development.

The insulin-signaling pathway is dependent on the lack of binding of insulin ligands to DAF-2. Under stable environmental conditions, insulin ligands bind to DAF-2, activating it and causing it to activate AGE-1 and other associated protein kinases. Of these protein kinases, AKT-1 phosphorylates DAF-16 resulting in cytoplasmic localization of DAF-16 and an increase in reproductive development. When external conditions are not optimum, the DAF-2 is not activated resulting in nuclear instead of cytoplasmic localization of DAF-16 and entry into dauers [148]. Gene families encoding insulin signaling pathway components are heavily

expanded in *C. elegans* and while some key components are conserved in parasitic species, the number of insulin ligands is drastically reduced in parasitic species (this thesis, [147, 149]).

The TGF-ß pathway is controlled by DAF-7. DAF-7 is expressed when environmental conditions are stable and allow continued reproductive growth [150]. Once activated, it binds to a DAF-1/DAF-4 complex, resulting in the phosphorylation of SMAD proteins DAF-8 and DAF-14, which inhibit DAF-5 and DAF-3 [141]. This prevents dauer development. DAF-3 also acts as a feedback loop, repressing DAF-7 and DAF-8 under dauer producing conditions [151]. DAF-7 is also activated when dauer larvae are transferred to fresh plates suggesting it is also involved in exiting the dauer stage [152]. The TGF-ß pathway is heavily conserved in parasitic species, with only the SMAD proteins appearing to be lost from clade I [147].

The outputs of these three pathways are then integrated by the nuclear hormone receptor signaling pathway and DAF-12. These are discussed in detail in the next section.

Recent studies have also begun to identify genes present in parasitic species that are involved in the production of infective larvae [149]. However, similar genetic and genomic analysis has also revealed that some of the genes controlling the development of dauer larvae in C. elegans are not present within parasitic nematodes [153] and that conserved genes present between free living and parasitic species can have different functions [141, 147]. Understanding the genetic nature of parasitism may be possible through a synthetic biology approach recently suggested by [14]. However, this work would require free-living species amenable to genetic transformation techniques, which are closely related to parasitic species of interest, species of which are currently lacking. The recent characterisation of dauer larvae in *Rhabditophanes KR. 3021* (as part of this thesis), a closely related relative of *Strongyloides spp*, suggests that this may be possible in the future.

- **The role of *daf-12* and dafachronic acid in dauer and infective larvae development**

As mentioned previously, steroid hormone signaling is one of the key pathways regulating development and dauer arrest. The most downstream gene of this pathway, *daf-12*, encodes a nuclear hormone receptor, which has had its natural ligand discovered (dafachronic acid (DA)) [154]. Its name giving function (daf: dauer formation defective), The effects on dauer formation of mutating either the receptor or the exogenous application of DA are identical, namely the inhibition of dauer formation [155] indicating that ligand binding inhibits the dauer promoting function of DAF-12. *daf-12* has a heavily conserved DNA binding domain [156-158] between different parasitic nematodes. Under favourable environmental conditions, dafachronic acid binds *daf-12* to stimulate reproductive growth [158]. When conditions are unfavourable, *daf-12* is unbound, resulting in the production of dauer larvae [159]. *daf-12* also acts as a convergence for pathways regulating developmental age and adult longevity [156, 159]. It is known to have functions in the developmental pathways and at various time points during the ontogeny of the worm [160]. It interacts with *daf-16* [161, 162] and *din-1* [163, 164] to control developmental events, including mediating the immune response with *let-7* microRNAs [165]. *daf-12* is implicated in metabolism, for example the production of cytosolic NADPH or fat metabolism [71, 158, 162, 166]. Interestingly in the context of parasites, *daf-12* is also known within *C. elegans* and *Strongyloides* to affect the response to temperature stress [71, 72, 167].

Application of dafachronic acid to free living generation of *S. stercoralis* and *S. papillosus* prevents L3i arrest, resulting in the formation of rhabditiform L3 and L4 in *S. stercoralis* [158], and a second generation of free living larvae in *S. papillosus* [168]. Dafachronic acid modulates the post-parasitic switch. L1 stages of S*. stercoralis* freshly excreted from a host when treated with DA, have a complete cessation of the direct development pathway resulting in all larvae undergoing free living development [169]. With the first successful native knockdown of *daf-12* in *S. ratti* ([71], part of this thesis), it was confirmed that

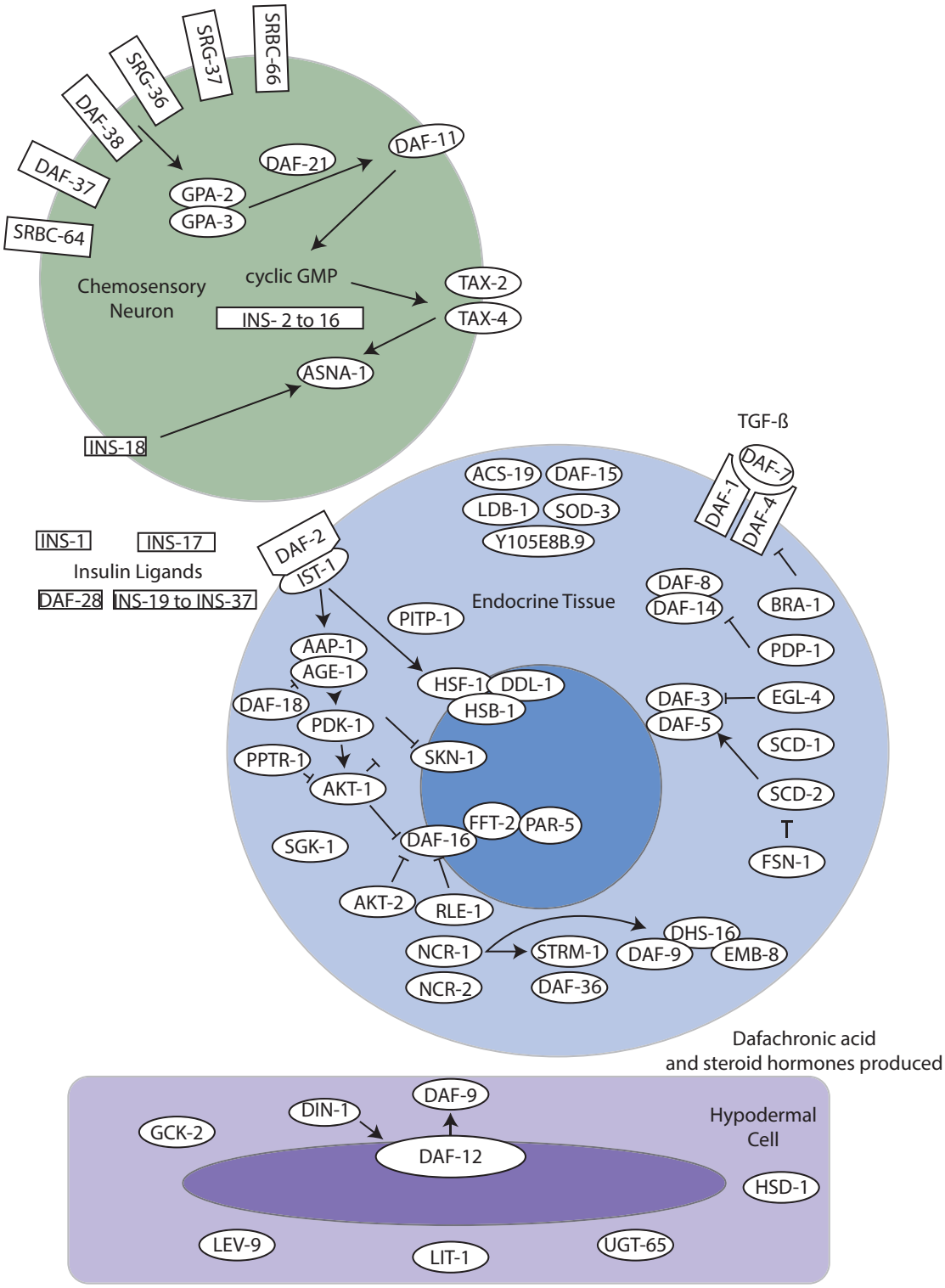


Figure 11: **Schematic representation of dauer pathway in *C. elegans*.**

Overview of proteins known to be involved in dauer signaling in *C. elegans* and

daf-12 functions were heavily conserved among distantly related nematodes and that DA is the natural ligand of *daf-12* in *Strongyloides*. Knockdown of *daf-12* resulted in the suppression of the direct development pathway and a reduction in infective larvae formation and infectiveness ([71], part of this thesis). Surprisingly given the conserved nature of dafachronic acid activity, the ligand-binding domain is not well conserved between different nematodes [170, 171]

The fact that life cycle choices can be regulated by the application of a steroid ligand, suggests that dafachronic acid and *daf-12* are good targets for the development of new antihelminthic drugs. The conservation of this pathway between phylogenetically distant nematodes like *Strongyloides* spp [71, 168, 169], *C. elegans* [168, 172] and *A. caninum* [158, 171], means this novel antihelminthic would have broad range of potential parasitic species that it could be used to target.

Aims of this thesis

The aims of this thesis was to understand fundamental processes involved in the production of infective larvae from both the direct and indirect development pathways and their relationship with dauer larvae, while also increasing the toolkit available for work on Strongyloididae species. This thesis can be considered to be split into four aims:

1) Method development within *Strongyloides ratti*

Strongyloides ratti is an attractive model organism for studying parasitism, as it not only has alternating free living and parasitic generations, but it also can be maintained within its natural host. However, the worms themselves are present within fecal cultures, which makes staging and experimentation difficult. Previous attempts at culturing the worms within a laboratory setting (such as on plates) have been unsuccessful as there is a complete loss of fecundity and survival. To counteract this, I generated a novel plate culture method that provides significantly improved survival and fecundity, making experimentation on these worms easier. Following this, I implemented genetic techniques within this species. As *S. ratti* appears refractive to microinjection, I developed a novel

soaking method for RNAi. This RNAi technique can be used across multiple life stages, appears to have minimal off-target effects and can be used to wide a range of genes.

2) **To understand infective larvae development (direct development) and the role of *daf-12***

Using the aforementioned RNAi technique, I studied infective larvae development in the context of *daf-12*. As *daf-12* is known to be involved in dauer formation and its ligand dafachronic acid is known to block infective larvae development in *Strongyloides*, I aimed to test whether dafachronic acid was truly acting through *daf-12* in *Strongyloides*, and whether infective larvae development would be truly suppressed. This represented the first native study of this gene within *Strongyloides*. I found that by suppressing *daf-12* expression, the direct development pathway was blocked and larvae had to instead develop through the indirect development pathway. Interestingly, the formation of infective larvae following sexual reproduction was not prevented, however there were developmental delays and significantly fewer larvae could develop to become infective larvae. These larvae were still able to infect a new host however the infection peak was both shortened and reduced in magnitude. I found that there is also a corresponding decrease in fecundity and interestingly a switch from aerobic to anaerobic metabolism within these larvae. Finally, these infective larvae have reduced thermotolerance. Together these results confirmed that dafachronic acid is acting through *daf-12* within *Strongyloides*, that the pathway is heavily conserved and that *daf-12* is a promising target for novel antihelminthics.

3) **Identifying new free-living species phylogenetically close to parasites of interest for the dauer hypothesis**

The dauer hypothesis states that dauers and infective larvae are homologous and that dauers serve as a pre adaptation towards parasitism. However, work on this important hypothesis has been stymied by a lack of free-living species close to parasites of interest, and in particular, free living species that make dauer larvae. *Rhabditophanes KR3021* is the closest free living relative to the *Strongyloides* genus of parasites. When observing the species, I noticed that it produced not only dauers, but also arrested J2 larvae in response to starvation,

neither of which had been previously reported. I therefore characterized the clearly lacking basic biology of this species, proving that the dauer larvae were "true" dauer larvae. I demonstrated that the *daf-12* pathway was conserved within this species, and showed the expression pattern of known dauer genes within this species by RNAseq. Finally, I characterized a novel fecundity strategy within the species.

4) **Understanding infective larvae development resulting from sexual reproduction (indirect development)**

It is known that only female larvae result from the sexual free-living reproductive event, yet it is not understood why. It is known that different species of *Strongyloides* produce different amounts of nullo-X sperm, but it is unclear how this results in no male progeny. The aim of the work was to characterize in detail the process of spermatogenesis in Strongyloididae and compare *P. trichosuri*, which does produce males and two species of *Strongyloides*, which do not produce males. To this end, novel and modified staining techniques had to be developed.

RESULTS AND DISCUSSION

This section consists of two published manuscripts, an unpublished manuscript and some unpublished results. .

Synopsis - Published Manuscripts

Optimizing culture conditions for free living stages of the nematode parasite *Strongyloides ratti*

Alex Dulovic, Vadim Puller and Adrian Streit

Experimental Parasitology. 2016 Sept: 168: 25-30.
doi: 10.1016/j.exppara.2016.06.005. Epub 2016 Jun 19.

SYNOPSIS

Alternating sexual free-living and parthenogenetic parasitic generations in addition to unique biological features make *Strongyloides* an attractive model system for studying parasite biology and evolution. Of these, the model parasitic nematode *Strongyloides ratti* (*S. ratti*) is particularly attractive as it can be maintained within its natural host [47, 173].

While this is beneficial, the free-living stages can only be extracted from fecal cultures making experimentation difficult. As these worms are known to be bacteriovorous, previous attempts were made to culture *S. ratti* using established *C. elegans* protocols (Nematode Growth Medium agar (NGM) and *E. coli* Op50 as a food source [174], however *S. ratti* unlike other species of *Strongyloides* is not amenable to these conditions. I therefore aimed to develop a novel fecal-free culture for *S. ratti*, allowing easier laboratory maintenance for experimentation, and increasing the toolkit within this species. Through altering the plate composition, food source, incubation temperature and presence of microorganisms, I generated a plate culture method with strongly improved fecundity and survival, allowing easier experimentation. This culture method

now allows easier access to worms for experimentation, makes staging of different life stages easier, and can be used to further increase the toolkit within this species.

CONTRIBUTION

I designed and performed all experiments with input from Adrian Streit and I analyzed the results with computational support from Vadim Puller. I wrote the manuscript with Adrian Streit. I consider my contribution to this study to be approximately 80%.

RNAi-mediated knockdown of *daf-12* in the model parasitic nematode *Strongyloides ratti*

Alex Dulovic and Adrian Streit

Plos Pathogens. 2019 Mar 29;15 (3):e1007705.
doi:10.1371/journal.ppat.1007705. eCollection 2019 Mar

SYNOPSIS

Strongyloides ratti (S. ratti), whilst being a model organism for understanding basic parasite biology and in the development of novel therapies, has a lack of forward and reverse genetic tools available. While CRISPR/Cas9 directed genome editing has recently been reported for *Strongyloides stercoralis (S. stercoralis)* [70], the authors found that CRISPR, much like other techniques involving the use of microinjection, work far less efficiently if at all in *S. ratti* compared to *S. stercoralis* [70]. To address this, I aimed to develop an RNAi by soaking protocol, to increase the available tools in this species whilst simultaneously eliminating the need for microinjection. After firstly determining by bioinformatic analysis that *S. ratti* had the requisite proteins for RNAi to potentially work, I developed the first successful RNAi by soaking protocol for *Strongyloides ratti*. While it had previously been thought that *Strongyloides* were refractive to RNAi [85], by changing to using short siRNAs instead of long dsRNAs, I was able to develop a protocol that results in consistent silencing of

genes of interest. After evaluating the effectiveness of the protocol across a range of genes and different life-stages of the parasite, while also determining the optimum soaking length required and the amount of off-target effects, I performed the first successful RNAi study in any species of *Strongyloides*. *daf-12* has long been known to be involved in the production of dauers within *C. elegans* [175], and as the morphological similarities of dauers and infective larvae results in them being considered homologous [141], this gene is of high interest in the development of novel antihelminthics [162]. Previous research on *daf-12* has also identified its ligand in *C. elegans*, dafachronic acid (DA), the application of which can not only prevent the development of dauer larvae in *C. elegans* and *P. pacificus* but also of infective larvae in *Strongyloides* spp. [168, 169]. However, it was not clear if the pharmacological effects of DA in Strongyloides spp. really occurred through the gene identified as the *daf-12* ortholog.

After suppressing *daf-12* expression by RNAi, I characterized the phenotypic changes present in *S. ratti*. As expected, suppression of *daf-12* severely impaired the formation of infective larvae in the direct development pathway, resulting in the generation of free-living adults. While *daf-12* suppression did not prevent the formation of infective larvae resulting from sexual reproduction (as part of the indirect development pathway), the offspring that were formed were developmentally delayed or unable to become the third stage larvae required for the invasion of a new host. Surprisingly, these developmentally delayed larvae were still able to infect a new host when injected subcutaneously, however the resulting infection was significantly reduced in terms of its burden and longevity. *daf-12* suppression also affected metabolism within infective larvae, causing a shift from aerobic to anaerobic metabolism. Finally, I found that *daf-12* suppression resulted in a loss of thermotolerance. Together these results provide not only a significant methodological breakthrough but also confirm the highly conserved nature of *daf-12* and its role in the formation of infective larvae, making it a viable target for the development of novel specific antihelminthic drugs.

CONTRIBUTION

I designed and performed all experiments with input from Adrian Streit. I performed all method development and phenotypic assays. I performed all the statistical analysis myself and generated all figures and tables with input from Adrian Streit. I mainly wrote the manuscript with contributions from Adrian Streit. I consider my contribution to this study to be approximately 90%.

Synopsis - Unpublished Manuscripts

Unpublished manuscripts are summarized now in the same form as above and then printed in full following the unpublished results. There is 1 unpublished manuscript that forms part of this thesis.

Rhabditophanes diutinus, a Parthenogenetic Clade IV Nematode that makes dauer larvae

Alex Dulovic, Tess Renahan, Adrian Streit

SYNOPSIS

The dauer hypothesis itself states that infective larvae evolved from dauer larvae, which represented a preadaptative stage for the evolutionary transition to parasitism, and that therefore dauer larvae and infective larvae are homologous [141]. In order to study this transition from free-living to parasite, we need well-studied closely related free-living species to parasites of interest, of which we are currently lacking [14].

Rhabditophanes KR.3021 is a free-living nematode that is closely related to the *Strongyloides* genus (dependent on the particular phylogenetic study, the two taxa belong to the same superfamily or even the same family). Some earlier authors had suggested that *Rhabditophanes* spp. is secondarily free living, after loosing the parasitic option of a facultative parasitic life cycle comparable to *Parastrongyloides* spp. Inspite of other *Rhabditophanes* species having dauer larvae described, no dauer larvae had ever been reported for *Rhabditophanes*

KR.3021, which is otherwise the best studied species of *Rhabditophanes*. When examining starved plates, I noticed that dauer larvae, by morphological criteria, and a second previously unreported stage of arrested J2 larvae (J2A) were present on the plate. I therefore aimed to characterize this species and its life cycle and provide clearly lacking basic information. The two starvation induced specialized larval stages appear to represent alternative survival strategies, one long term (dauer) and one short term (J2A). Further, I found that this species varies its reproductive rate and duration dependent on the population density. With respect to the dauer hypothesis and the possibility that *Rhabditophanes* is secondarily free-living, it was of particular interest to determine if the *Rhabditophanes* KR.3021 dauer larvae were indeed the homologous stage to *C. elegans* dauers and *Strongyloides* infective larvae. The dauer larvae showed all of the morphological characteristics of *C. elegans* dauers, including a buccal plug that allowed them to survive SDS treatment. I confirmed that the dauers were third stage larvae as are C*. elegans* dauer and *Strongyloides* spp. infective larvae. I identified orthologs of genes present within the *Rhabditophanes* KR.3021 genome known to be involved in dauer larvae production in *C. elegans*, and showed that, with this respect, *Rhabditophanes* KR.3021 resembles strongly *Strongyloides* spp. I showed that dafachronic acid (DA), the ligand of the nuclear hormone receptor DAF-12 in *C. elegans*, prevented the formation of dauers (but not J2As) in *Rhabditophanes* KR.3021 as it does with dauer larvae in *C. elegans* and infective larvae in *Strongyloides* spp. This indicated that the conserved DAF-12 regulatory module involved in *C. elegans* and *Strongyloides* spp. dauer/infective larvae formation is also involved in *Rhabditophanes* KR.3021 dauer formation. Taken together these results strongly suggest that *Rhabditophanes* KR.3021 dauer larvae and *Strongyloides* infective larvae are indeed homologous. This makes it rather unlikely that *Rhabditophanes* reverted from parasitism and regained a dauer stage and renders *Rhabditophanes/Strongyloides* a highly attractive system for further study of the dauer hypothesis. Finally, I identified orthologs of genes present within the *Rhabditophanes KR.3021* genome known to be involved in dauer larvae production in *C. elegans*, and compared them to the genes present within *Strongyloides ratti* and *papillosus*. Together this work provides a

comprehensive, detailed description of the life cycle and morphology, in addition to reporting several important characteristics present within this species that is now an attractive system for use in studying parasitism. As a result of the multiple survival strategies that this species displays, we have proposed renaming the species from the species code *KR3021* to *Rhabditophanes diutinus*.

CONTRIBUTION

I designed all experiments with input from Adrian Streit. I performed all experiments apart from the SDS treatment of dauers. I performed all statistical analysis and generated all figures and tables. Adrian Streit and myself wrote the manuscript. I consider my contribution to this study to be around 80%.

Unpublished results

Strongyloides eliminates male-determining sperm through spermatogenesis

A genetic system whereby females have two X chromosomes and males one (XX/X0) appears to be the most common within nematodes, and is possibly the ancestral system [6]. As discussed in the introduction, among the Strongyloididae genus, *Strongyloides* appears to have transitioned from GSD to ESD relatively recently [46], as seen by the impact of host immune status upon the sex ratio of the offspring produced by the parasitic female [47-49, 176]. The chromosomal XX/X0 configuration is still present and in the closely related species *Parastrongyloides trichosuri* still serves for GSD [39, 41]. The life cycle of Strongyloides (Figure 3) results in only female offspring from a sexual reproduction event. This elimination of males is unclear and not uniform to the Strongyloididae family as a whole. For instance, in the closely related parasitic species *P. trichosuri,* we know that males are also formed as a result of this sexual reproduction event, yet we have seen that is in a reduced ratio compared to females. It is unclear how exactly males are eliminated from *Strongyloides* offspring. However it must be relevant to their biology and a recent development, given how *Parastrongyloides* still produces males.

Previous research within the lab by quantitative sequencing data and cytology had suggested that some, but less than the expected 50%, nullo X sperm and inviable XO early embryos are formed within *S. ratti* but not *S. papillosus* [127]. In order to address how the formation of male progeny is prevented I examined spermatogenesis within the Strongyloididae. While spermatogenesis has been well characterized within the model species *C. elegans* it is poorly understood within nematode parasites. The few nematode spermatogenesis studies outside of *C. elegans* have already revealed novel strategies resulting in highly distorted sex ratios [117, 176]. For example, the free living species *Auranema rhodensis* (previously described as *Rhabditis* sp. SB347) operates a highly unusual spermatogenesis pathway whereby a modified meiosis step results in male determining (nullo X) sperm being used as a residual body, blocking their development and resulting in 95% female progeny [117].

Primary spermatocyte formation

While the overall structure of the gonad of Strongyloididae has been previously determined ([125], Figure 9), it was unclear whether a rachis like structure was present from which primary spermatocytes detached as it is the case in *C. elegans.* To determine how spermatogenesis begins in this family, young *P. trichosuri* males were observed live under DIC microscopy (Figure 12).

This revealed that primary spermatocytes in fact detach from the bottom of the entire germline syncytium (hereon referred to as giant cell). Following condensation of chromosomes after the small nuclei, primary spermatocytes cellularise and then separate from this nuclei and then begin to divide. To confirm the lack of a rachis like structure and that this was consistent within the family, dissected gonads of *P. trichosuri*, *S. ratti* and *S. papillosus* were stained with phalloidin, which stains filamentous actin. Phalloidin staining (Figure 13) confirmed that the primary spermatocytes and dividing cells were held in place by actin bridges and the internal pressure of the gonad instead of a rachis like core.

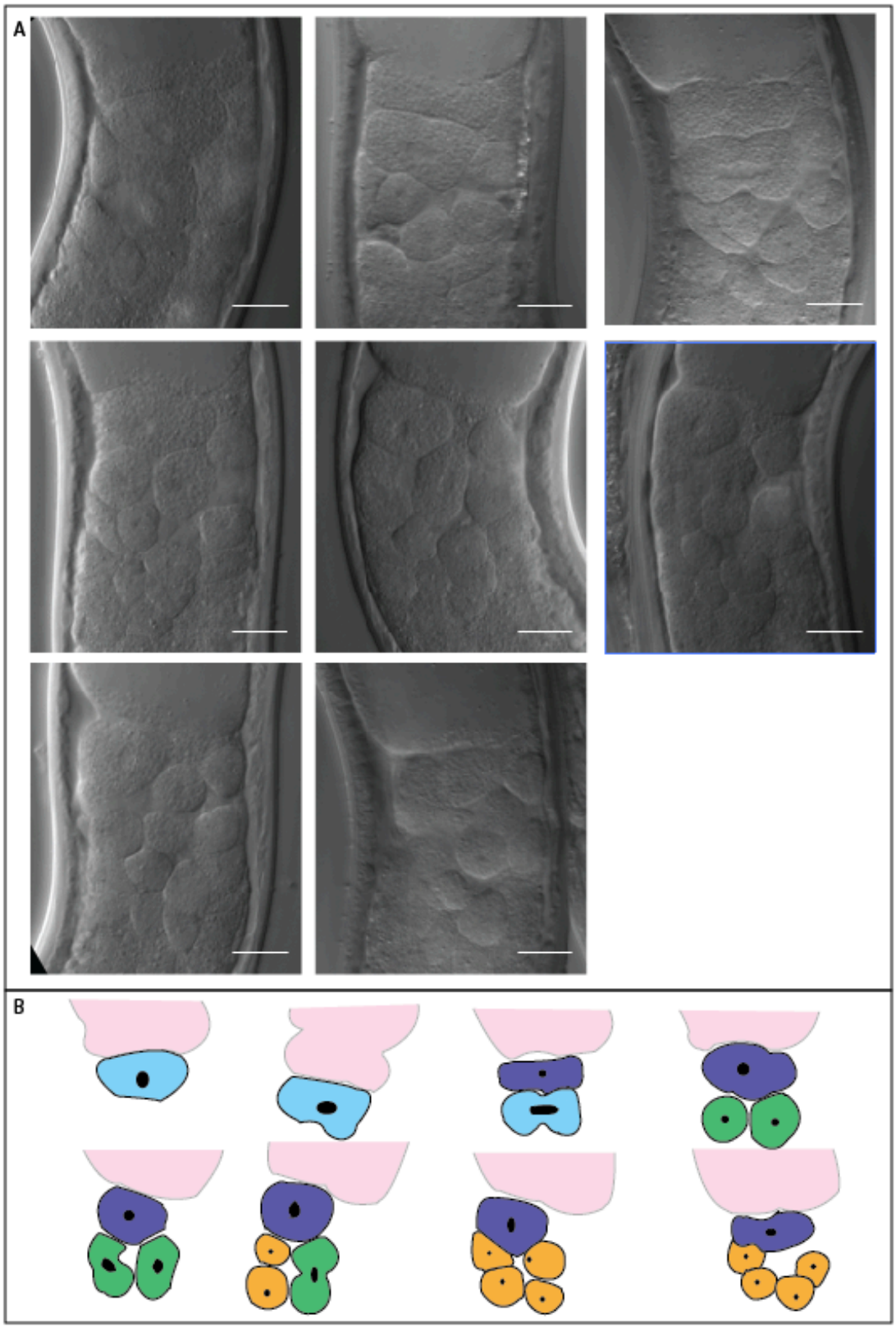


Figure 12: **Live Imaging and Illustration of Spermatogenesis in Strongyloididae**. (A) Time course of spermatogenesis observed under live DIC in *P. trichosuri*. (B) Illustration of the above time course. A primary spermatocyte (blue), divides into two secondary spermatocytes (green), which

in turn divide into spermatids (orange). A new primary spermatocyte (purple) separates from the giant cell (pink).

Interestingly it also showed that the small nuclei also appear to be held in place by or at least have actin bridges between them.

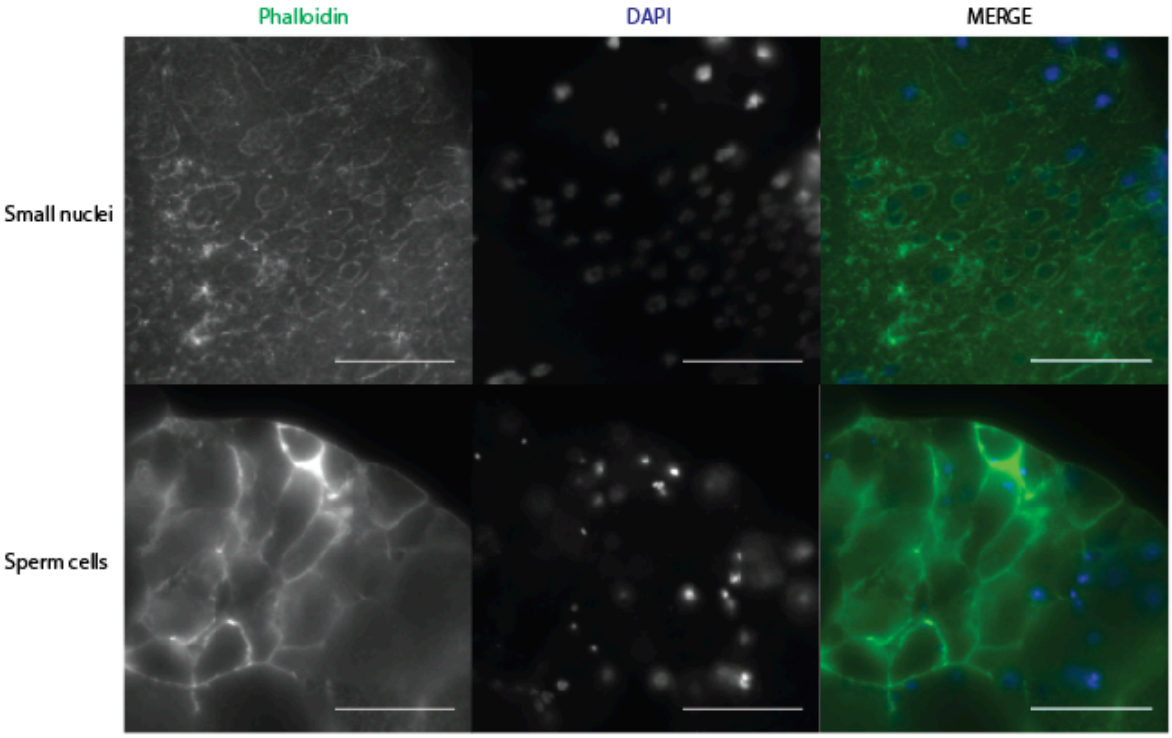


Figure 13: **Phalloidin staining of *Strongyloides ratti* gonad**. The gonad including small nuclei appear to be linked by actin bridges, while sperm cells are held together by actin bridges instead of a core rachis like structure. Scale bars are 20µm.

Within the giant cell, chromosomes appear to condense and separate into groups, where they appear to divide and reproduce once before separating into primary spermatocytes. As seen in Figure 14, the giant cell is substantially larger and contains multiple dividing nuclei as indicated by the presence of tubulin spindles. It is unclear whether this early division represents an initial duplication to produce greater numbers of cells, or is necessary to generate the chromosomes required for the primary spermatocytes to bud off into.

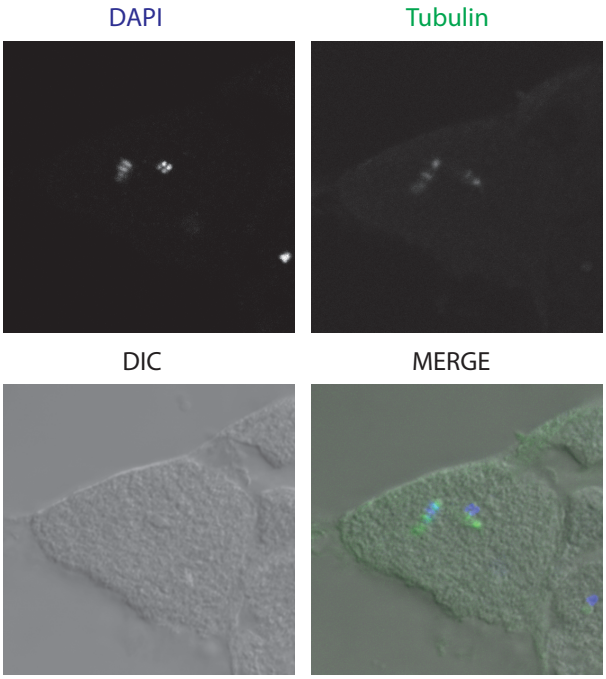


Figure 14: The Giant cell contains dividing chromosomes. Giant cell of P. trichosuri. It is considerably larger than all other spermatogenesis cells and contains multiple dividing chromosomes, as indicated by the presence of tubulin spindles.

Spermatogenesis in Strongyloididae

After determining the absence of a rachis, and seeing that primary spermatocytes were generated from the giant cell. To characterize spermatogenesis and specifically the divisions involved, I characterized spermatogenesis within *S. ratti*. By using glyoxal fixation instead of paraformaldehyde [177], I found that improved resolution could be seen for both tubulin spindles and DAPI signal. As seen in Figure 12, there are two meiotic divisions following the formation of the primary spermatocytes, resulting in the generation of four spermatids from the original primary spermatocyte. During the first meiotic division, the X chromosome does not divide and instead segregates with one pair of autosomes, resulting in one secondary spermatocyte receiving 3 chromosomes (2 autosomes and the X) and one secondary spermatocyte receiving 2 chromosomes (2 autosomes only) (Figure 15).

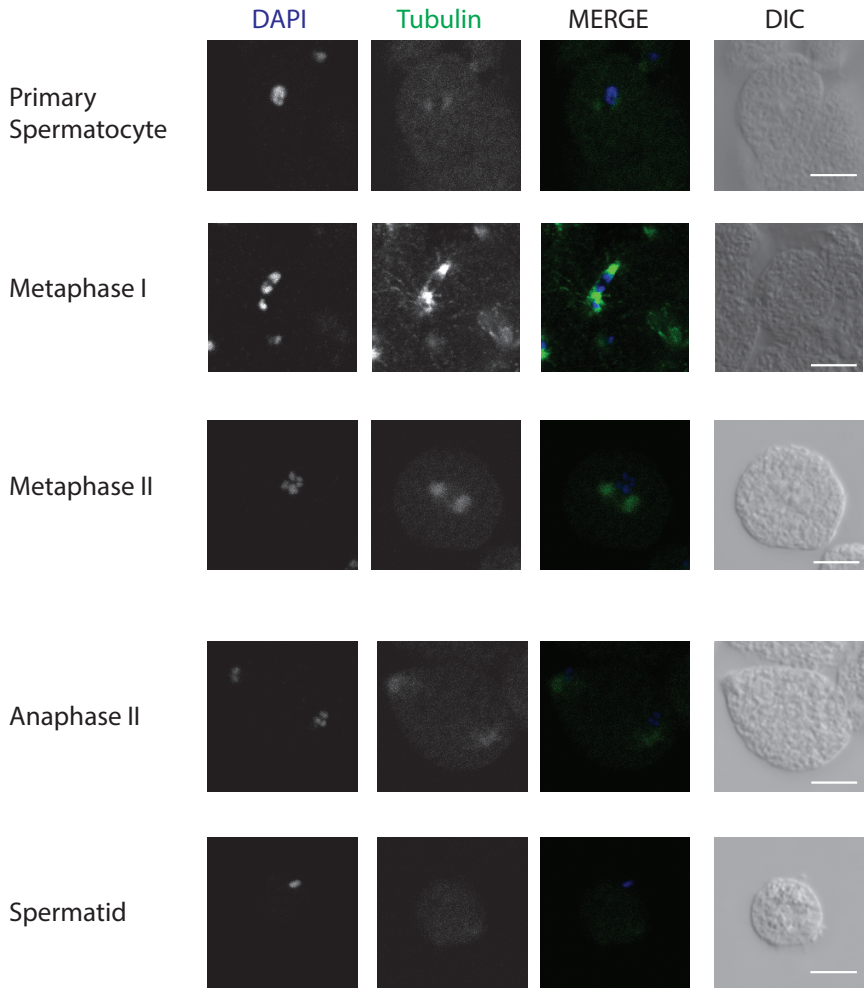


Figure 15: **Spermatogenic divisions in *S. ratti***. Spermatocytes undergo two divisions to form a spermatid. During the first division, the X chromosome segregates to a single pair of autosomes. Scale bars are 5µm.

The male determining (nullo X) pair however appears to be non-functional. In the same location as spermatids and dividing secondary spermatocytes within the gonad, we see the appearance of residual body-like structures. These residual bodies have a different appearance to the rest of the spermatocytes, appearing shiny under DIC (Figure 16). We assume that these residual bodies are derived from the nullo-X cells as we do see spermatocytes without an X chromosome from that point on in the gonad. Within these residual bodies themselves, we see tubulin and major sperm protein (MSP) staining, along with the absence of any DNA signal. Interestingly we found these residual bodies in all 3 Strongyloididae species.

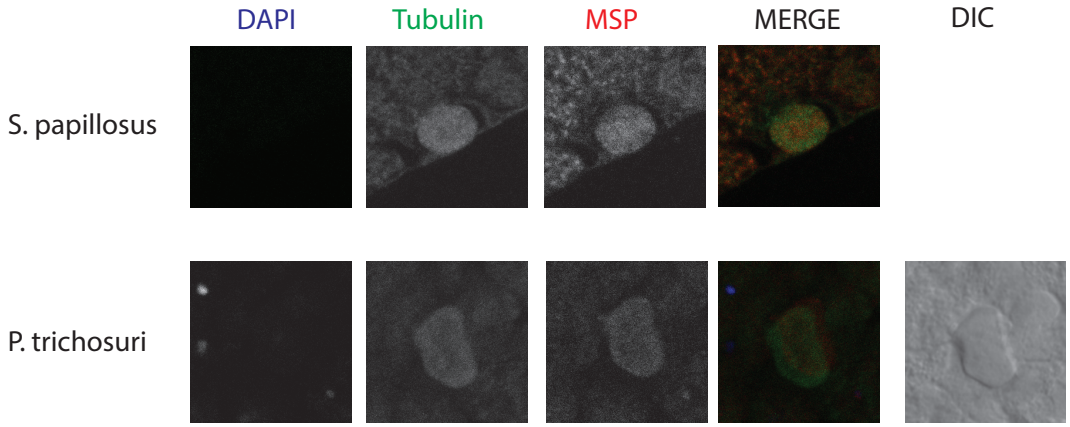


Figure 16: **Residual bodies within the Strongyloididae**. Residual bodies are found within all 3 species, however only S*. papillosus* and *P. trichosuri* are shown here. They contain protein staining, no DNA signal, and have a different appearance under DIC to spermatocytes.

To confirm the identity of the X chromosome and to follow it in nuclei where the chromosomes are not visibly separated in *S. ratti*, fluorescent *in situ* hybridization (FISH) was performed on dissected gonads. As seen in Figure 17, during the first meiotic division from primary to secondary spermatocytes, a single signal corresponding to the X chromosome can be seen, which is transferred only to one of the daughter cells. When we examine secondary spermatocytes, we see some cells that have a signal, and some that don't, indicating the presence of X-bearing and nullo-X cells. During the second meiotic division, in female determining sperm, we see an equal division of the chromosome, with both spermatids receiving an X chromosome. Similarly, we see dividing secondary spermatocytes that have no signal, indicating the absence of an X chromosome. Finally, when examining sperm, we see that all sperm has a signal, meaning that only female determining sperm are being formed.

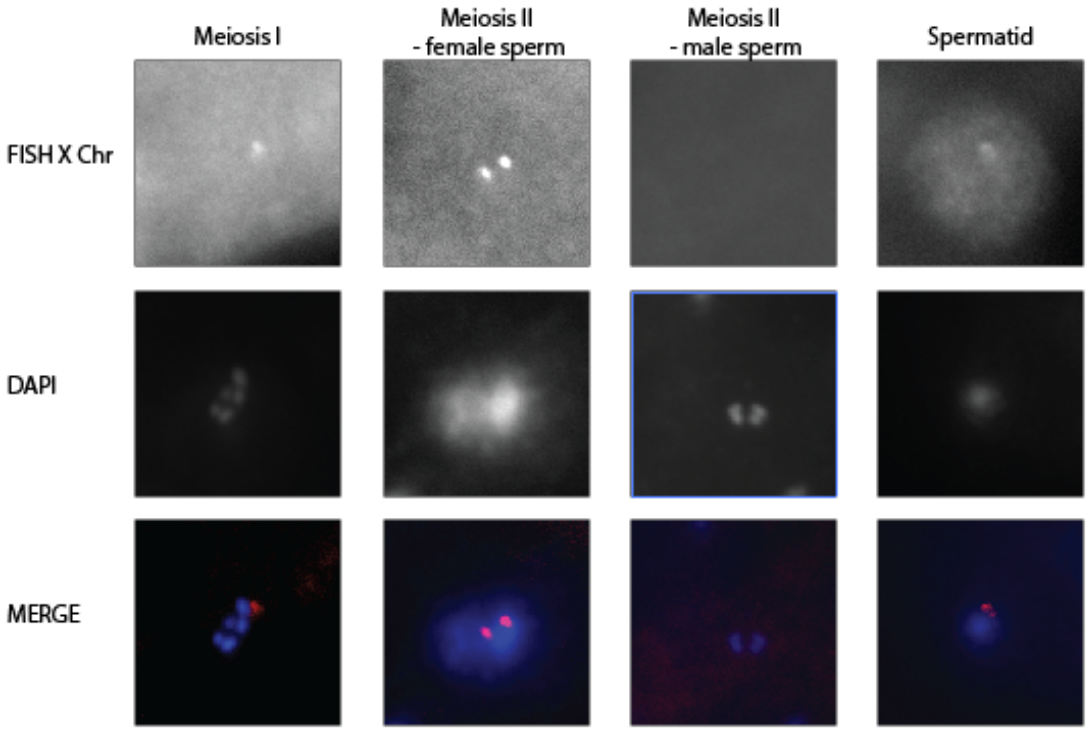


Figure 17: **FISH of X chromosome in *S. ratti***. Dissected gonads were stained with FISH probes against the X chromosome, confirming that the X chromosome segregates during the first meiotic division. During meiosis II, this results in secondary spermatocytes that have X chromosomes and separate, and those that don't. When examining sperm cells we see that all have an X chromosome suggesting that male-determining sperm are non-viable.

Identification of Fibrous Bodies-Membranous Organelles (FB-MOs) in *Strongyloides ratti* and Major Sperm Protein (MSP) expression

MSP has a characteristic expression pattern when examining stained gonads (Figure 18A). There is expression through the entire gonad, but when examining primary spermatocytes and secondary spermatocytes, we see they have a distinct punctate staining pattern. This is likely due to MSP being expressed as the primary spermatocytes separate from the giant cell. Later in spermatids, there is consistent localization pattern on the membrane, which then becomes polar as the spermatids mature. This expression pattern indicates that msp plays a crucial role in the production of sperm. To understand this expression pattern in more detail and to determine if it was correlated with meiotic divisions, we examined gonads under Electron Microscopy. We found that MSP

is stored within FB-MOs as in *C. elegans,* however that these FB-MOs are far smaller and far more numerous than in *C. elegans* (Figure 18B). We found that FB-MOs in *S. ratti* contain multiple chambers, with a double envelope membrane, that retracts when MSP is being expelled (Figure 18C). To determine what was being secreted by these FB-MOs, we performed immunogold staining on EM sections and found that all the fibers present appear to be MSP (Figure 18D). As seen in Figure 18D, gold particles are localized only to cells with external fibers and not to the empty chambers, suggesting that msp is sole constituent of the FB-MOs.

Conclusion

We found that the spermatogenesis pathway is highly conserved among Strongyloididae but not compared to *C. elegans*. The absence of a rachis like structure suggests profound differences between these phylogenetically distant species like *C. elegans* and *S. ratti*, and that even basic processes such as spermatogenesis are not highly conserved. We found that in *S. ratti* nullo-X spermatocytes are formed by Meiosis I, and are still undergoing Meiosis II, but then appear to be transformed into residual bodies. It is possible that some nullo-X sperm may be able to avoid this process, hence explaining why sequencing and cytological results suggested the presence of some nullo-X sperm and non-viable early embryos with a male karyotype within *S. ratti*. It remains unclear and requires further work to determine what the role of the mitotic division is in the giant cells. Similarly, further stainings and particularly FISH need to be performed on *P. trichosuri* and *S. papillosus*, to determine their meiotic divisions. We also require better understanding of gonad formation within *Strongyloides*, as it is still unclear the different functions that large and small nuclei have, and how even the small nuclei are formed. With this we may be able to understand further how this division event occurs, and what its significance is in terms of reproduction. It should be noted that *Strongyloides ratti* appears to employ a similar strategy to that reported for *Auranema rhodensis* [117], in that male determining sperm appear to be used by female determining sperm as residual bodies, suppressing their development.

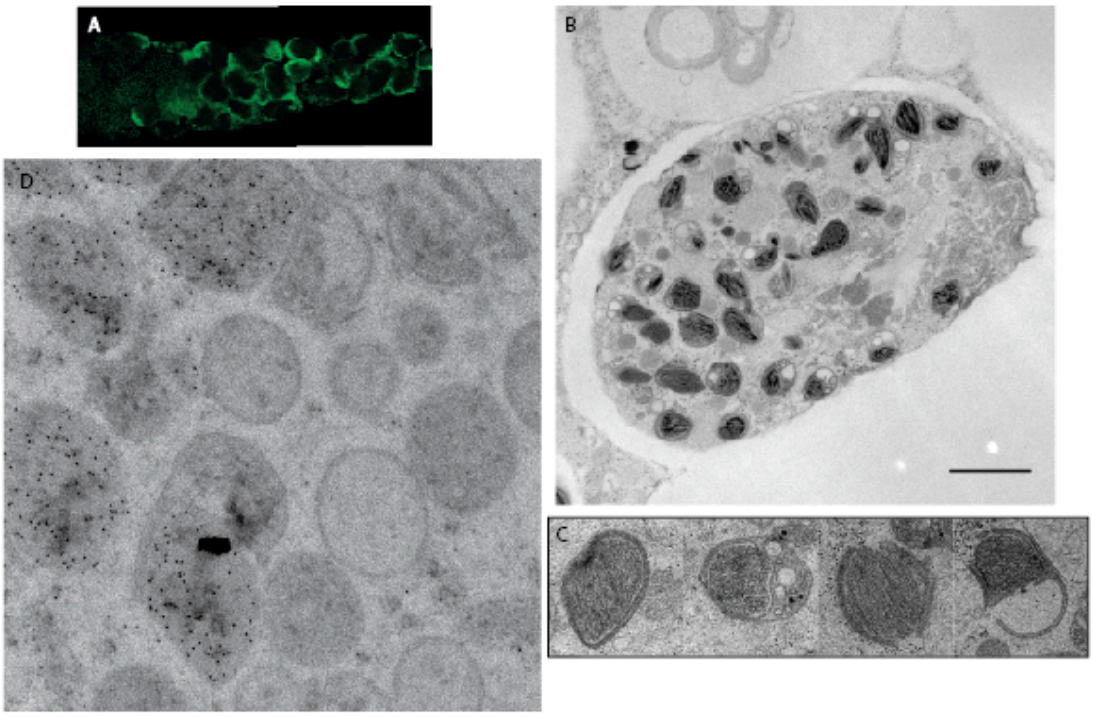


Figure 18: **Major Sperm Protein expression and Ultrastructure in *Strongyloides ratti***. Clockwise from left: (A) Major Sperm Protein stained gonad showing localization pattern from punctate in primary spermatocytes to polar in sperm. (B) Presence of MSP in Fibrous Body – Membranous Organelles in an *S. ratti* spermatocyte. (C) FB-MOs consist of multiple chambers filled with sperm cells, the membrane of which later retracts to allow the MSP to be released resulting in empty chambers. (D) Immunogold staining confirms that FB-MOs consist of major sperm protein as seen by the presence of black dots on chambers still with fibers present compared to those without. Scale bar in B of 2µm.

Methods - Worm Strain and Cultures

Strongyloides ratti strain ED321, a derivate of ED5 [178] was obtained from M. Viney, Univ. of Bristol, in 2010 and has since been kept in continuous culture in our in-house animal facilities as described [178] with occasional reverting to frozen stock in order to minimize in lab evolution. Briefly, 4 week old Wistar rats (Charles River) are injected subcutaneously with around 500 infective larvae and allowed to development for 7 days. Following this, the rat feces is collected overnight and cultured at 19°C for 2 to 3 days to allow development to males. The worms are then harvested from the fecal cultures by Baermann funnel as

described in [179]. *Strongyloides papillosus* isolate LIN [29] was maintained in-house in female New Zealand White rabbits as described [29]. Briefly, about 1.5 kg rabbits are injected subcutaneously with about 2000 infective larvae and development allowed for 12 days, after which rabbit feces is collected overnight, mixed with sawdust and moistened to increase humidity, and then incubated at 25°C in a humid incubator for 2 to 3 days to allow development to adults. The worms are then harvested by Baermann funnel. *Parastrongyloides trichosuri* strain QA414 [39]is a 12-times inbred derivate of a wild isolate originally obtained from Warwick Grant, AgResearch, New Zealand. It was maintained as permanent free-living culture on NGM plates with a piece of autoclaved rabbit feces supplemented with *E. coli* OP50 as described [41]. *C. elegans* N2 was obtained from the *C. elegans* Genetics Center at the University of Minnesota and maintained on NGM plates supplemented with Op50 as described [174].

Methods - Live Observation of Strongyloididae

For live observation, all species of Strongyloididae were picked onto a drop of water on a 4% agar pad and visualized under DIC Microscopy on a Zeiss Imager Z1 with a Zeiss Axiocam mono camera as in [180].

Methods - Immunofluorescent Stainings

For all antibody stainings, males were transferred into 10µl of sperm salts [180]on a Poly-L-Lysine coated slide and dissected by cutting the pharynx with a needle, causing the gonad to release. 10µl of Glyoxal fixing solution [177] (for Tubulin) or 2% PFA (for MSP) was added, mixed briefly up and down by pipetting and then a coverslip placed on top, gently pressure applied and incubated for 4 minutes. Slides were then frozen in liquid nitrogen, the coverslip removed with a scalpel, and then incubated in -20°C Methanol for 1 minute. Slides were then transferred into room temperature PBST (1.5% Triton X-100) for 30 seconds before being blocked in 0.7%BSA PBST for 1 hour. Following this, 50µl of antibody staining solution was added to the slides, a coverslip placed on top, and incubated overnight in a humidity box at room temperature. The following morning, slides were washed 3 times for 5 minutes each in PBST, and then 50µl of secondary antibody staining solution was added to each slide, a

coverslip placed on top, and incubated in darkness in a humidity box at room temperature for 5 hours. Following this, slides were washed 3 times for 5 minutes each in PBST in the dark, the remaining solution wicked off with a KimWipe paper, and 10µl of 1µg/ml DAPI in VectaShield added, a coverslip placed over the top and sealed with nail polish. The slides were then stored in the dark overnight to allow the DAPI stain to permeate the sample and then examined either on a Zeiss Imager Z1 microscope or Leica SP8 Confocal Microscope.

The following primary antibodies were used: Tubulin (DM1A, Sigma, 1:100), FITC-conjugated Tubulin (DM1A, Sigma, 1:50), Major Sperm Protein 63197(1:10000), 63198 (1:10000), 4A5 (1:5000) and 4D5 (1:5000) (all gifts of D. Greenstein, Univ. Minnesota [181]

The following secondary antibodies were used: Alexa488 (Thermo 1:100), Cy3 (Thermo 1:100), Alexa 637 (1:100)

Phalloidin staining followed the protocol described in [180] except that Glyoxal was used instead of PFA to fix the samples. Phalloidin was Alexa488-conjugated (Molecular Probes). Slides were imaged on a Zeiss Z1 Imager Microscope.

Methods - FISH

Clustered repeat sequences on the X chromosome of *S. ratti* were identified using Tandem Repeats Finder [182]. Sequences that had multiple consecutive copies (at least 10) were considered and ordered as 20 to 50nt length oligos from Eurofins Genomics with a 3´modification of Cy3. The ordered probes were diluted with H20 to 100µM and then diluted 1 to 100 in Hybridization Solution (170µl 20X SSC, 122.5µl H_20, 580µl Formamide and 0.1275g Dextran). Males were then isolated and their gonads dissected out as described above into 10µl of Sperm salts on a poly-l-lysine coated slide. 10µl of Sperm salts containing 0.12% Triton X-100 was added, mixed up and down by pipetting, 10µl removed and incubated for 2 weeks. 10µl Glyoxal solution was added, mixed up and down by pipetting, 10ul removed, a coverslip added and incubated for 5 minutes. The slides were then frozen in liquid nitrogen, coverslip removed with a scalpel and incubated overnight in -20°C Methanol. The following morning, the jar was removed from the freezer and allowed to warm upto RT for 15 mins. Following

this, slides were washed twice in 2X SSCT (0.8% Tween 20) for 5 mins each, and then incubated overnight in 2X SSCT/Formamide at 37°C. The following morning, the slides were removed and allowed to air dry until most of the liquid had evaporated but the gonad had not dried. 10µl of hybridization solution containing the probes was added, a coverslip placed on top, and sealed using rubber cement. The slides were stored in the dark for 10 mins whilst the rubber cement dried. The slides were then incubated on a heat block at 96°C for 2 mins, and then incubated overnight in darkness in a humidity chamber at 37°C. The following morning, slides were removed, the rubber cement removed, and washed three times for 5 minutes each in 2X SSCT in the dark. 10µl VectaShield containing DAPI was added, a coverslip placed on top and sealed with nail polish. The slides were then stored overnight in darkness and then examined on Zeiss Imager Z1 microscope. The following sequences were used as probes: AAACAAAAAATATTTATCATTATTAAAATTAGTGATAAATAAC, ATGATATAAAATCTTCATTTGACT and AATTCATCAGAAACTTCTGG. All probes were mixed together before use.

Methods - Electron Microscopy

Male *S. ratti* adults were isolated as described above, and picked 20µl of H_20 in a 1.5µl Eppendorf and delivered to the in-house Electron Microscopy facility. For Immunogold staining, the 63198 Antibody was used.

Methods - Image Analysis

All images were analyzed using Fiji. At least 40 gonads were examined from each species. Images were resized using Photoshop and Figures were then generated in Illustrator.

Rhabditophanes diutinus a Parthenogenetic Clade IV Nematode with Dauer Larvae

Alex Dulovic[1], Tess Renahan[1] and Adrian Streit[1]*

[1]Department of Integrative Evolutionary Biology, Max Planck Institute for Developmental Biology, Tübingen, Baden-Württemberg, Germany

* Corresponding author

E-mail: adrian.streit@tuebingen.mpg.de

Short title: *Rhabditophanes diutinus*

Abstract

A lack of free-living species that are phylogenetically close to parasites of interest, is one of the main obstacles towards studying the evolution of parasitism and the nature of this lifestyle itself. Within Clade IV of the nematode phylum, parasites of the genus *Strongyloides* are of much research interest due to their life cycle and other unique biological features. *Rhabditophanes* sp. is their closest known non-parasitic relative, which was however speculated to be secondarily free-living. Recently, the genomes the Strongyloididae (four species of *Strongyloides*, the facultative parasite *Parastrongyloides trichosuri* and the free-living species *Rhabditophanes* sp. KR3021) were sequenced, providing insights into the genomic nature of parasitism. Although *Rhabditophanes* sp. KR3021 was included in this comparative genomic work, very little is known about this species. Based solely on morphology, dauer larvae had been described for other species of Rhabditophanes but so far not for *Rhabditophanes* sp. KR3021. Upon examination of starved plates, we identified dauer larvae and another previously unreported stage of arrested J2 (J2A) larvae within this species. Further examination revealed that the dauer larvae had all of the characteristic morphological features of *C. elegans* dauer larvae and could survive SDS treatment. Further we showed that dafachronic acid (DA) inhibits the formation of dauers, as it does in *C. elegans*, showing also that aspects of genetic control are conserved. This strongly suggests that dauers in *Rhabditophanes* sp. KR3021 are homologous with *C. elegans* dauer and *Strongyloides* spp. infective larvae and that *Rhabditophanes* is a very interesting taxon to study in order to evaluate the so called dauer hypothesis for the evolution of nematode parasitism. J2A larvae were unable to survive SDS

treatment and their formation was unaltered by DA, suggesting that the represent a separate survival strategy, controlled by a different genetic pathway. We also found a novel fecundity strategy within the species to ensure its prolonged survival, and identified orthologs of known dauer genes present within its genome.

Keywords: *Rhabditophanes* sp. KR3021, *Rhabditophanes diutinus*, meiotic parthenogenesis, dauer, dafachronic acid, germ line, *daf-12*, parasitism

1. Introduction

One of the main obstacles towards gaining a deeper understanding of parasitism, the evolution thereof and the specific parasites themselves, is a lack of well-studied closely related free-living species. The widely studied model nematode *Caenorhabditis elegans* (*C. elegans*), whilst a highly tractable model organism with an extensive basis of knowledge, is too far evolutionarily removed from many parasitic species of interest [1, 2]. *C. elegans* itself also appears to be a non-representative example of its genus, given that there are substantial differences in genome size and genome number within the *Caenorhabditis* genus [2]. This limits the possible gain of knowledge about parasites by comparing them to *C. elegans*.

For the clade IV nematodes [1], which contain the highly important *Strongyloididae* parasites, the evolutionary distance between themselves and *C. elegans* make even basic comparisons difficult. For instance, it is known that *C. elegans* and *Strongyloididae* produce different small RNAs [3] and have had different gene families expand [4]. The necessity of having more closely related free-living nematodes to parasites of interest is particularly stark given that phylogenetic analysis indicates that parasitism has arisen at least 200 times across 15 phyla [5], to which nematodes are no exception. In the phylum nematoda itself, the five major clades [1] all of which contain parasites, have seen parasitism evolve on upto 18 separate occasions [2]. A recent review [2] posited several hypothesis to explain why nematodes have so easily and

frequently made the jump to parasitism . Without closely related free-living model organisms, we will never be able to answer fundamental questions in parasites such as the very genetic nature of parasitism, and in particular the dauer hypothesis.

The dauer hypothesis assumes based upon similarities in morphology, physiology, genetic control, behaviour and role, that dauers serve as a pre-adaptation to parasitism [6]. Dauers themselves are a long lived life stage found in some nematodes [7]. In *C. elegans*, these arrested larvae are produced after the second molt in response to harsh environmental conditions (overcrowding, lack of food, unsuitable temperature), allowing the population to survive [8]. Once the environment has normalised, they are able to exit the dauer stage and recover [7]. Dauer formation in *C. elegans* is governed by four signaling pathways: cyclic GMP (cGMP), TGF-ß, Insulin signaling and the steroid hormone signaling pathway [6, 9]. Of these, the steroid hormone signaling pathway is the most downstream, and directly regulates dauer formation and recovery through *daf-12* [6, 10-12]. For a detailed overview of dauers and the genetic pathways involved in their development within *C. elegans*, we encourage the readers to consult [6-8]. By comparison, parasitic nematodes such as the Strongyloididae make infective larvae as part of their life cycle (a comparison between the *C. elegans* and Strongyloididae life cycles can be found in figure 1). Infective larvae of Strongyloididae have long been considered to be homologous to dauer larvae based upon morphology and their long survival time compared to the more rhabditid stages of their life cycle [13]. Like dauers they are third stage larvae, contain a buccal plug, have a radially restricted body plan and have a minimally

98 developed gonad [8]. They also exhibit similar behaviours such as nictation and
99 the cessation of pharyngeal pumping [8, 14]. However, it is currently unclear
100 whether dauers and infective larvae are truly homologous or not. It is known
101 that their formation can be altered through the steroid hormone signaling
102 pathway of *daf-12*. Suppression of *daf-12* function by mutation, RNAi or the
103 supplementation of dafachronic acid (the natural ligand of DAF-12 in *C. elegans*)
104 results in a loss of dauer development in *C. elegans* and *P. pacificus* and to
105 redirection of infective larvae development to the formation of free-living stages
106 in both generations of various species of *Strongyloides* [10, 11, 15-19]. This
107 suggests that a conserved endocrine regulatory module around DAF-12 controls
108 dauer formation in *C. elegans* and L3i formation in Strongyloididae, which might
109 be indicative for a common evolutionary origin. Recent studies have also begun
110 to identify genes present in parasitic species that are involved in the formation of
111 infective larvae [20]. However, similar genetic and genomic analysis also reveal
112 that other genes involved in the controlling the development of dauer larvae are
113 not present within parasitic nematodes [21], and that genes conserved between
114 free-living and parasitic species can even have different functions [9, 22].
115 Complicating this further, studies involving isolating and disrupting genes in
116 parasites have been slow due to the lack of and success of available tools,
117 although this has recently started to change [15, 23]. A recent review paper [2]
118 also suggested a synthetic biology approach towards understanding parasitism
119 by converting a free-living nematode into a parasitic nematode, instead of
120 disrupting genes of interest within a parasitic nematode due to the likely
121 macroevolutionary problems and high probability of inducing a lethal
122 phenotype.

The approach of using genetic and genomic analysis to identify genes and other parasitism-associated features has already begun to pay dividend with the discovery of acquired gene families coding for astacins, SCP-TAPS proteins, acetylocholinesterases and prolyl endopeptidases in parasitic *Strongyloides* species [4]. The Strongyloididae themselves are interesting for studying the evolution of parasitism as they contain taxa with a diverse range of lifestyles including facultative parasites (*Parastrongyloides* spp.), obligate parasites (*Strongyloides* spp.) and free-living nematodes (*Rhabditophanes* sp.) [4]. The phylogeny of these taxa can be found in figure 2. *Rhabditophanes* spp. is the closest non-parasitic relative of *Strongyloides* spp. and as such of particular interest with respect to the dauer hypothesis for the evolution of parasitism at the specific example of *Strongyloides* spp. [24]. Phylogenetic analysis [25] had previously suggested that *Rhabditophanes* spp. is secondarily free-living upon losing the parasitic option of a facultative parasitic life cycle as is still present in *Parastrongyloides* spp. However, this conclusion was largely based on the erroneous phylogenetic placement of *Rhabdias bufonis,* which has in the meantime been corrected [26]. Among the different species of *Rhabditophanes*, *Rb*. sp. KR3021 is the one for which the highest amount of molecular and cellular information is available and it is considered a "Tier 1" species by the International Helminth Genome Consortium [27]. Different from other species of *Rhabditophanes* [28, 29], *Rb*. sp. KR3021 reproduces parthenogenetically [4] and no males have ever been described. Much of the previous research on *Rhabditophanes* sp. KR. 3021 has been highly tissue specific cytology or was purely genomic, resulting in cytological knowledge of its embryonic cell lineage

148 [30], vulva development [31], intestinal lamellae [32] and gonad structure [33]
149 and the availability of fairly high-quality genome assembly [4]. However there
150 remains a complete absence of knowledge about its basic biology. There exists
151 no formal species description and no species name. In order to provide some
152 much needed information about this free-living relative of parasites, we set
153 about to characterize various aspects of the *Rhabditophanes KR. 3021* biology
154 with special emphasis on the life cycle, in particular dauer formation. We found
155 that this species under starvation conditions, produces a low level of dauer
156 larvae.,We found that these dauers represent an equivalent life cycle stage to
157 those found in *C. elegans*, can be easily recovered with the addition of food, and
158 that this dauer stage is far longer living than the other stages of the life cycle. To
159 our knowledge, this is the first case of a free-living clade IV species, which can be
160 induced to enter and exit a dauer stage in a laboratory setting. We also noted that
161 *Rhabditophanes* produces a far larger amount of arrested J2 larvae (J2A). These
162 larvae are not dauers, nor can they develop to dauers. However, while they
163 survive starvation for longer than regular J2s, they do not survive as long as
164 dauers. This indicates that they provide a short term survival strategy for the
165 species compared to the long term survival strategy of making dauers. We also
166 found that *Rhabditophanes* changes its reproductive output in response to
167 crowding and sensing its own offspring. Finally, we identify the homologs of
168 known *C. elegans* dauer genes in *Rhabditophanes* and show that the *daf-12*
169 pathway is highly conserved in this species as was previously thought. Together
170 with the data shown, we also provide a morphological description for the adult,
171 dauer and arrested larval stages of *Rhabditophanes* (as this was previously
172 lacking). Based upon the data shown in this paper and the importance of the

species overall, we suggest renaming the species from *Rhabditophanes KR. 3021* to *Rhabditophanes diutinus* reflecting its longevity and multiple strategies to increase survival, as well as the long time from its isolation to naming. By providing increased knowledge of a free-living species that is far more closely related to parasites of interest than standard model nematodes, we believe that several outstanding questions about parasitism can be addressed, such as what the genetic and genomic changes underlying the transition from free-living to parasitic species are, and what the role of dauer arrest is in the evolution of parasitism [2].

2. Results

2.1 Life Cycle of *Rhabditophanes diutinus*.

Previous descriptions of *R. diutinus*.(KR3021) state it has a simple free living cycle consisting of four larval molts from embryos through to adults with reproduction occurring by meiotic parthenogenesis [4](Figure 3A). For other species of *Rhabditophanes*, but not for *R. diutinus*, the existence of dauer larvae associated with arthropods had been described, solely based on morphology [28, 29]. When examining overgrown *R. diutinus* plates, we saw worms of two previously undescribed stages: a dauer-like larvae (Figure 3B) and a small arrested stage larvae (Figure 3C). If the hypothesis is true that *Rhabditophanes* spp. is secondarily free-living [25] and derived from a parasite originally arisen by the transition of the dauer larva to an infective larva (dauer hypothesis), one would expect that *Rhabditophanes* spp. has lost its original dauer stage. From this one could speculate that if a functional dauer stage exists, then this has been newly gained after the reversal to non-parasitic life style. In order to determine if

198 *R. diutinus* does indeed form dauers that are the homologous stages of dauer
199 larvae of clade V nematodes, we further investigated the two starvation induced
200 stages.
201 First, we treated mixed overgrown cultures with SDS [14]. In *C. elegans,* dauer
202 larvae are the only developmental stages outside of the egg shell that survive this
203 treatment. Resistance to SDS is due to both a thickened cuticle and buccal plug,
204 which insulate the dauers from their environment [14, 34]. In *R. diutinus* only the
205 dauer-like larvae were able to survive the treatment and could be recovered on
206 an NGM plate (although it should be noted that they took substantially longer to
207 recover than *P. pacificus* or *C. elegans* dauers, see below). To determine whether
208 these dauer-like larvae were morphologically similar to *C. elegans* dauer larvae,
209 larvae were examined under DIC microscopy for a buccal plug. As seen in figure
210 3D, these larvae contain a 3 part buccal plug consisting of a plug in the mouth, a
211 further larger plug located approximately one third of the way down in the
212 intestinal lumen(Fig 3E), and a final series of plugs located throughout the
213 posterior part the lumen, indicating that like their *C. elegans* counterparts, they
214 do not feed.
215 The arrested small stage larvae (hereon referred to J2A) could not survive SDS
216 treatment. Microscope examination revealed they lack a buccal plug or any other
217 distinguishing features of dauer larvae and that they are most similar to J2 larvae
218 (Figure 3F). However, they are capable of surviving far longer than any normal
219 J2 larvae (see below) and only occur when a plate is starved and has run out of
220 food sources. To determine if they were an earlier developmental stage for
221 dauer larvae, J2A larvae were transferred onto either bacteria free NGM plates or
222 *E. coli* OP50 supplemented NGM plates to determine if they would develop into

dauer larvae or what stage they would recover at. No J2A developed into dauer larvae suggesting they are an alternative to and not a part of the dauer formation pathway. When recovered with food, the J2A larvae developed into morphologically normal looking J3 larvae further suggesting that this is a independent survival pathway from dauer larvae. This suggests that *R. diutinus* contains multiple strategies for survival when under environmental stress.

2.2 Dauer and J2A Recovery

As J2A and dauer larvae appear to represent different survival strategies for *R. diutinus* larvae, we firstly wanted to determine at what exact stage the different larvae are formed. As it was impossible to mount and track L1 larvae for long enough to allow them to undergo differentiation, we instead recovered dauer and J2A larvae on NGM plates supplemented with Op50 and observed their recovery to adults. As seen in figure 4, dauer larvae undergo two molts from dauers into J4 and J4 into adults showing that dauers are third stage larvae, as in *C. elegans* [7]. Interestingly, it takes nearly 2 hours for the buccal plug to disappear and a further 4 hours for the intestinal plugs to be removed, perhaps explaining why the recovery following SDS treatment took longer than in *P. pacificus* or *C. elegans*. By contrast, the J2A larvae required 3 molts to become adults, which took nearly 24 hours longer than in dauers. This, in addition to the fact that J2A can not form dauers confirms that they represent different survival strategies. An updated version of the life cycle of *R. diutinus* can be seen in figure 3G.

2.3 Identification of orthologs of known dauer genes in R. diutinus

To determine if genes known to be involved in the development of dauer larvae of *C. elegans* are present within *R. diutinus*, all genes listed in [6, 20] were searched for in the *R. diutinus* published genome [4]. As seen in table 1, only for 64 out of the 102 *C. elegans* genes, clear orthologs are present in *R. diutinus*. Of the genes present, about half are 1 to 1 orthologs. Interestingly, we noticed a striking difference in conservation between different signaling pathways involved in dauer formation in *C. elegans*. The vast majority of these missing genes are from the insulin-signaling pathway, in particular insulin ligands. By contrast in the TGF-ß pathway, 12 out of the 13 *C. elegans* genes have orthologs in R. sp. KR3021, 9 of which are 1 to 1 orthologs. This itself is also in complete contrast to the cGMP pathway, where despite 10 of the 12 genes being present within *R. diutinus*, only 3 are 1 to 1 orthologs. When compared to the closely related *Strongyloides* species, *Strongyloides ratti (S. ratti)* and *Strongyloides papillosus (S. papillosus),* we see that all species have similar genes present within their genomes (Figure 5). As seen in Figure 7, 51 genes are conserved between all three species, with *R. diutinus* having more genes present (64) than either *S. ratti* (52) or *S. papillosus* (55). However most of these extra genes present in *R. diutinus* come from the Insulin signaling pathway which is even less conserved within *Strongyloides*. An illustration showing the conservation of dauer genes between *C. elegans* and the Strongyloididae and their role in *C. elegans*, can be seen in figure 6.

Table 1 - Genes known to be involved in the dauer signaling pathway in *C. elegans* that are present within the published *Rhabditophanes diutinus* genome.

Pathway	**Total Genes in *C. elegans* pathway**	**1 to 1**	**1 to Many**	**Many to 1**	**Many to Many**	**Missing**	**Total Present in *R. diutinus***
cGMP	12	3	5	0	2	2	10
TGF-ß	13	9	0	0	3	1	12
Insulin	63	16	2	9	3	33	30
Steroid Hormone	14	5	5	2	0	2	12
Total	102	33	12	11	8	38	64

Genes are grouped according to the pathway in which they belong, and whether they are 1 to 1, 1 to Many, Many to 1 or Many to Many orthologs compared to *C. elegans*

2.4 Dafachronic acid prevents dauer formation but does not prevent J2A formation

To test whether the *daf-12* pathway is conserved within *R. diutinus*, larvae were treated with dafachronic acid (DA) or ethanol as a negative control and their developmental stage monitored 10 days later as in [10]. No dauer larvae were seen when plates were supplemented with DA indicating that dauer development has been prevented by the DA (table 2). However, there was no difference in the formation of J2A larvae (95.92% in the negative control compared to 94.35-94.48% when treated with DA) indicating that their development is regulated by a different pathway (table 2).

Table 2 – Dafachronic acid treatment prevents the formation of Dauer but not J2A larvae.

	Treatment		
Worm Stage (%)	**Ethanol**	**10µM Δ7 DA**	**100µM Δ7 DA**
Dauers	2.60	0.0 (**)	0.0 (**)
J2A	95.92	94.35	94.48

Young larvae were transferred on new plates supplemented with either Δ7 dafachronic acid or ethanol (solvent) and incubated for 14 days following which the amount of dauer and arrested J2 larvae were counted. On each plate, 200 larvae were chosen at random and staged. On plates were no dauer larvae could be counted within the 200, the whole plate was examined to determine if it truly was free from dauer larvae or not. For each treatment, 5 plates were used, and the experiment was repeated three times. Data shown here is a mean of all samples. An ANOVA was carried out to determine statistical significance between the different treatments (p = 0.003 for both

Taken together the results presented so far, we conclude that R. diutinus forms dauer larvae which most likely are homologous to dauer larvae of *C. elegans* and to L3i's of *Strongyloides* spp.

2.5 Dauer and J2A Survival

To determine how long both sets of larvae could survive, dauers and J2A were transferred separately onto bacteria-free NGM plates and observed daily for upto 3 weeks. As a control, normal J2/J3 larvae were also included. As seen in figure 7, J2/J3 larvae had the shortest lifespan (average of 3.99 days) with all the larvae dead after 12 days. Interestingly, the transfer of these larvae into a foodless

environment resulted in them stopping their development. The J2A larvae were able to survive significantly longer than the J2 larvae (p-value 0.017) with an average lifespan of 5.45 days, but all had died after 14 days. As expected, the dauer larvae had a significantly longer lifespan than both the J2 (p-value <0.0001) and J2A larvae (p-value <0.0001) with an average survival of 9.91 days for those that perished over the course of the experiment, and 24% were still alive at the end of the 3 weeks. It is currently unknown how long these dauer larvae may be able to last for but they have been seen in plates for upto 3 months.

This data suggests that dauer larvae provide a long term survival strategy for *R. diutinus* whereas the J2A larvae provide a much shorter survival strategy.

2.6 Transgenerational effects of being dauers

Next we asked if the progeny of individual who underwent dauer development are more likely to form dauers themselves. Such an effect could either be caused by genetic differences within the population predisposing some genotypes for dauer development, or it could be due to an epigenetically encoded transgenerational effect. To this end, J2A and J2/J3 were transferred in groups onto NGM plates supplemented with Op50 and incubated at 15°C. After 2 days of producing offspring, the initial worms were removed from the plates and the total number of offspring produced counted. As seen in Table 3, there was no difference in the number of offspring produced between J2/J3s and recovered dauers and J2A (78.5 for J2/J3, 73.9 for dauer and 73.8 for J2A). After further incubation for 2 weeks, the percentage of dauer larvae present on each plate was then counted. There was no difference in dauer production between J2/J3s

(2.95%), recovered J2A (2.89%) and recovered dauers (2.92%). These results suggest that the larvae undergoing dauer development are not genetically different and the there are no transgenerational effects present from being dauers, at least none that perdure for longer than one generation.

Table 3 – No transgenerational effects of being a dauer or J2A larvae compared to a J2/J3 in terms of offspring produced and likelihood to become dauers in the next generation.

Worm Stage Plated	Offspring after 48h of laying	Dauers after 14 day incubation (%)
J2/J3	78.5 ± 7.25	2.95 ± 0.35
Dauer	73.9 ± 5.30	2.89 ± 0.52
J2A	73.8 ± 4.80	2.92 ± 0.45

There is no difference in production of offspring or dauer formation between any of the starting populations. For each starting population, 10 plates were picked and the experiment was repeated three times. Data shown here is a mean of all samples plus the standard deviation. A students t test and Mann Whitney U were carried out to determine statistical significance between the different treatments, none was found.

2.7 Fecundity strategies

While there was no difference in fecundity between adults and recovered either J2A or dauer larvae, it was noticed that there a substantial difference in fecundity based upon how many larvae were initially plated. To confirm this, J3 were transferred onto fresh NGM plates with *E. coli* OP50 either singly or in groups of 5 or 10. They were then allowed to develop for 2 days until they started laying

embryos. The numbers of embryos laid along with any hatched offspring were then counted for 3 days. For some of the single worm plates, the adult was transferred daily to a new plate. As seen in figure 8, there is statistically significant decrease in the number of offspring produced on the first day among the different populations. Whilst there is no statistically significant difference between the single worm and 5 worm plates, there is a strong decrease between 1 worm and 10 worms, with worms maintained in groups of 10 producing nearly half as many eggs on average (14.49) as compared to worms alone on a plates (26.77) (p-value <0.0001). 24 hours later, this pattern of decreased fecundity in response to crowding still persists, with worms kept in groups of 10 producing 52.30 offspring on average compared to 82.29 offspring for groups of 5 and 90.39 worms for single worms (p-values both <0.0001). Most surprisingly however, there is a strong significantly significant decrease in the number of offspring produced between single worms kept on the same plate (90.39) versus those transferred to a new plate daily (123.16) (p-value <0.0001). 24 hours later this pattern remains, with single worms transferred daily having produced an average of 203.48 offspring compared to single worms kept on the same plate (188.10), worms kept in groups of 5 (167.22) and 10 (120.45). This strong correlation between crowding and a reduction in offspring, suggests that *Rhabditophanes* senses its own offspring and adjusts its reproductive output based upon how many offspring are already present.

3. Discussion

3.1 Dauer Production in *Rhabditophanes diutinus*

385 *Rhabditophanes diutinus* produces a very low amount of dauers in response to
386 starvation. It also produces arrested J2 larvae that are not as long lived as dauers
387 and are unable to become dauers, representing a second strategy for the long
388 term survival of the species in response to stress. To our knowlege, this study
389 provides the first example of a fully free-living Clade IV nematode that can be
390 induced to enter and exit a dauer stage under laboratory conditions. The
391 importance of this in the context of the dauer hypothesis cannot be understated.
392 The lack of closely related free-living nematodes to parasites of interest, is
393 continuing to stymie the understanding of the very nature of parasitism, in
394 addition to parasite development and evolution [2, 9, 35]. Only through the
395 study of nematodes like *Rhabditophanes* can this gap in knowledge begin to be
396 filled. It is unclear as to why dauers in this species have not been previously
397 reported, particularly seeing as dauers were originally reported for this genus
398 over 80 years ago [28].
399
400 **3.2 *Rhabditophanes diutinus* has multiple survival strategies**
401 The production of dauers is rare enough within Clade IV to warrant further
402 investigation of this species, let alone the existence of a second separate survival
403 strategy to environmental stress. *Rhabditophanes diutinus* appears to have two
404 different strategies to allow it to survive environmental stress such as lack of
405 food. While it is not uncommon for a nematode to have different survival
406 strategies, it is unexpected for the nematode to appear to favour the J2A pathway
407 over the dauer pathway (as based upon percentage of J2A over dauers).
408 Logically, we would expect the dauer pathway to be favoured given its superior
409 ability to survive environmental stresses [7]. The reasons behind this apparent

prioritization need further investigation. One possibility may be that worms need to feed in order to reach the dauer stage, whereas they might be able to reach the J2A stage based upon digestion of their yolk alone. We also need to subject the worm to further stresses (i.e. temperature, salinity, osmolarity, oxygen concentration) in order to see if this ratio of dauers to J2A changes in response to different environmental stresses.

3.3 Conservation of dauer genes in *Rhabditophanes* and *Strongyloides* provides insight into the dauer hypothesis

While genes can have alternate functions between different nematode species [22], *daf-12* and other genes involved in the steroid hormone pathway appear highly conserved in their function [10, 15, 17, 36]. The high conservation of certain genes and pathways is particularly promising for the dauer hypothesis as it suggests a shared origin, whilst missing genes between the species could potentially be crucial in the difference between dauer and infective larvae. One could speculate that perhaps loss of genes in the insulin signaling pathway may be a fundamental difference between infective larvae and dauers or that the Insulin signaling pathway might be involved in the formation of J2As. Interestingly, the steroid hormone pathway is conserved completely between the three species, so J2A formation may be more linked to either the Insulin signaling or an as yet unknown pathway.

The conservation of the genetic repertoire and the functional conservation of the DA - DAF-12 endocrine module provide strong support for the dauer hypothesis for the evolution of parasitism in the phylogenetic branch the led to

435 *Strongyloides* spp. Although we cannot exclude a perfect reversal, our data make
436 it unlikely that *Rhabtitophanes* is secondarily free-living. If it were true that *R.*
437 *diutinus* is secondarily free-living, we would not expect the species to have two
438 distinct survival pathways present. Similarly, if dauers had only recently been
439 re-acquired by the species, then it seems unlikely that known dauer genes such
440 as *daf-12* would still have the same function as is found in far evolutionarily
441 distant species like *C. elegans,* Further investigation of the dauer formation
442 control genes as identification of their target genes is warranted, particularly
443 through RNAseq to determine if expression of these genes is consistent between
444 free-living and parasitic species. RNAseq is also necessary to identify other
445 genes that are strongly up or downregulated in dauers that may be unique to this
446 species or to this clade. It should also provide insights into the difference
447 pathways involved in the development of dauer and J2A larvae which appear to
448 be different. Through further study of this species, we have an opportunity to
449 gain real understanding of the nature of parasitism. It is therefore also of the
450 upmost importance to develop forward and reverse genetic tools such as EMS-
451 mutagenesis and CRISPR so that we can start to knockout genes of interest and
452 understand their function in dauer formation and parasitism itself. It should be
453 noted however that as this species is parthenogenetic, we cannot ensure
454 successful transmission of mutations of interest into offspring, nor can we
455 backcross out mutations arising from screening methods such as EMS.
456

457 **4. Methods**

458 **4.1 Species and strain**

Rhabditophanes KR. 3021 was originally isolated by Ann M. Rose near Vancouver, British Columbia [31]. The strain was sent to our lab by Dee Denver (Oregon State University) in July 2014. It has been since maintained in the lab at 15°C on NGM plates supplemented with Op50 bacteria as a food source or as frozen stock (frozen according to [37]). This strain is the same as that in the recent *Strongyloididae* genome paper [4].

4.2 Staging of worms and determination of dauer molts

Worms were picked manually from mixed stage culture plates into 5µl of water on a 4% agarose slide and visualized under a microscope using DIC optics [38]. For the dauer staging, dauers were isolated from 2 week old plates that had been starved of food for at least a week and then visualized directly using DIC microscopy on an Zeiss Imager M2 microscope with a Zeiss Axiocam 506 mono camera. To determine the dauer recovery and number of molts, dauers were picked from the same plates onto fresh NGM plates supplemented with Op50 in batches of 5 and then incubated at 15°C. A plate was removed every 2 hours (upto 44 hours) and the worms on the plate were examined under 40x DIC microscopy to determine stage and changes in development between time points. High magnification images of the different stages was taken using a Zeiss Image Z1 with a Zeiss Axiocam 506 mono camera.

4.3 SDS Treatment of dauers and other stages

Old (minimum 2 weeks) plates that had run out of food supply and contained dauers, J2 arrest and a small number of long-living adults were washed off into SDS solution and incubated gently shaking for 20 mins. Following this, the

worms were washed multiple times with water and then recovered on NGM plates with Op50 at 15°C. 1 hour later, plates were examined for surviving larvae and their developmental stage was scored.

4.4 Dauer survival determination

Dauers, arrested J2s and J2/J3s were picked in groups of 5 onto NGM plates without any bacteria and incubated for 15 days at 15°C. The plates were checked daily and the number of surviving worms counted. Plates were picked in batches of 6 per life stage and the experiment was repeated three times.

4.5 Test for transgenerational effects from dauers

To examine for any transgenerational effects, dauers, J2A and J2/J3s were picked onto fresh NGM plates with a lawn of Op50 and incubated at 15°C for upto 15 days. Plates were checked daily until they began producing offspring. From this day, the original worms were allowed to lay embryos for a further 2 days at which point they were removed. Plates were checked daily to count the number of offspring produced. Once the worms had been removed, the plates were then incubated at 15°C for 10 days after which the amount of dauers and J2A were counted on every plate. Worms were picked onto plates originally in groups of 10, with 5 plates per life stage. This experiment was repeated three times.

4.6 Fecundity determination

To examine fecundity changes, J3 worms were picked either singularly, in batches of 5 or 10 onto fresh NGM plates with a lawn of Op50 and incubated for 48 hours at 15°C to allow development to adults. Following this, plates were

examined and total number of offspring (laid eggs and hatched larvae) were counted along with the number of adult worms still alive on the plate. For plates with multiple original larvae, the adults were left on the plate overnight before counting the offspring again. For plates with single worms, the adults were either left on the plate or transferred to a fresh plate and incubated again overnight before counting the offspring. This was repeated once more so that 3 days of offspring production were counted (total of 120h from the original picking of the J3s). 10 plates were picked per treatment, with this experiment being repeated three times.

4.7 Bioinformatics analysis of dauer pathway in *R. diurinus* and *S. ratti* and *S. papillosus*

All genes known to be involved in the formation of dauer larvae in *C. elegans* [6, 20] were searched for using the method described in [15, 39]. Briefly, genes were searched for in the *Rhabditophanes* KR3021 (PRJEB1297), *S. ratti* (PRJEB125) or *S. papillosus* (PRJEB525) genome by BLASTp using WormBase Parasite (WBPS14 (WS271) parasite.wormbase.org). All proteins that returned a blast score greater than E-5 were carried forward and their domains determined using InterProScan. Only those which contained the same domains as the *C. elegans* gene were considered to be the gene of interest. If a protein could not be found, then it was searched for using both its Protein and DNA sequence (BLASTx and BLASTn). If the gene was still not found then it was considered to be not present within the genome.

4.9 Dafachronic acid experiments

Dafachronic acid experiments were performed similarly to as described in [10]. *E. coli* OP50 was grown overnight in LB medium and then centrifuged and resuspended in 1/5th volume of 0.9% NaCl. 90µl of resuspended bacteria and either 10µl of 10 or 100µM Δ7 dafachronic acid or ethanol were combined and spotted on an NGM plate. L3 worms were added to the plate and incubated for 14 days at 15°C after which the plates were examined and all worms staged.

4.10 Statistical analysis and figure generation

Appropriate statistical analysis was carried out using Excel, with statistical significance determined as being reached once the p-value was below 0.05. The exact statistical test used is noted in the figure or table legends. Microscopy images were resized in Photoshop and then annotated in Illustrator. Figures were generated in Excel and Illustrator.

5. Acknowledgements

We thank Waltraud Röseler and Christian Rödelsperger for their help with this manuscript and all other members of the Department for Integrative Evolutionary Biology for useful discussions.

6. Figure Legends

Figure 1 – Life cycles of *C. elegans* and *S. ratti.* Stages marked with "i" must develop into parasitic females. It should be noted that in *C. elegans*, all stages can be hermaphrodites or males, whereas in *S. ratti*, all parasitic adults are parthenogenetic females, while the free-living adults are both male and female.

559 Figure 2 – Phylogenetic relationship of *Rhabditophanes* and selected
560 *Strongyloididae* species based on [2, 4]. Tree branch lengths are not indicative of
561 evolutionary distance among the species.
562
563 Figure 3 – Life cycle and developmental stages of *R. diutinus.* (A) previously
564 described simple life cycle according to [4]. (B) J2A larva. (C)dauer larva. (D)
565 buccal plug (arrow) and (E) the uppermost of several intestinal plugs (star). (F)
566 Front ends of J2A , lacking a buccal plug but featuring an ordinary narrow open
567 mouth (arrows). (G) Updated life cycle for *R. diutinus* .
568
569 Figure 4 – Recovery of dauers into adults in *Rhabditophanes sp. KR3021*. (A) Time
570 course of dauer recovery. Images are taken from a time course experiment.
571 Images are shown at 4 hours intervals from dauers (t=0 hours) through to fully
572 developed reproducing adults (t=44 hours). (B-D) High magnification DIC images
573 of the two molts, at 16 and 36 hours are shown. Scale bars indicate 100μm. The
574 images shown here are a composite of the experiment and do not constitute a
575 single worm as it develops.
576
577 Figure 5 - Homologs of genes known to be involved in the dauer signaling
578 pathway in *C. elegans*, within the *R. diutinus* (RSKR), *Strongyloides ratti* (SRAE)
579 and *Strongyloides papillosus* (SPAL) published genomes. Black – the species has
580 at least one ortholog of this gene present within its genome. White – the gene
581 could not be found within the species genome.
582

583 Figure 6 –Dauer formation in *C. elegans*. This figure is based upon those in [6, 9,
584 20]. Genes that are present within the Strongyloididae are in red. Genes present
585 in *R. diutinus* but not *S. ratti* or *S. papillosus* are in blue. Genes that are only
586 present within C. elegans are in black.
587
588 Figure 7 – Dauers survive longer than J2A or J2/J3 when on fresh foodless plates.
589 Survival curves of Dauers (dark blue line) J2A (green line) and J2/J3 (light blue
590 line) when transferred onto fresh plates to induce starvation. Per treatment, 120
591 worms were picked in groups of 5 and checked daily for survival. The
592 experiment was performed at three time points such that each time 40 worms
593 per treatment were analyzed in parallel. The data points shown here are the
594 daily mean of all 120 worms. Error bars are standard deviation.
595
596 Figure 8 – Box plot of fecundity over time for different densities. *R. diutinus* J2s
597 were picked either singularly (red), in groups of 5 (yellow) or 10 (green) onto
598 plates and maintained for 120 hours or picked singularly onto plates and
599 transferred daily to a new plate (blue). Offspring produced after 72, 96 and 120
600 hours was recorded. Anovas were performed between the different samples at
601 each time point. For each density, 10 plates were picked, and the experiment
602 was repeated three times. * indicates a statistically significant value of between
603 0.05 and 0.01, ** indicates a statistically significant value of between 0.01 and
604 0.001 and *** indicates a statistically significant value of lower than 0.001.
605
606 **7. References**

1. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, et al. A molecular evolutionary framework for the phylum Nematoda. Nature. 1998;392(6671):71-5. Epub 1998/03/24. doi: 10.1038/32160. PubMed PMID: 9510248.

2. Viney M. How Can We Understand the Genomic Basis of Nematode Parasitism? Trends in parasitology. 2017;33(6):444-52. Epub 2017/03/10. doi: 10.1016/j.pt.2017.01.014. PubMed PMID: 28274802; PubMed Central PMCID: PMCPMC5449551.

3. Holz A, Streit A. Gain and Loss of Small RNA Classes—Characterization of Small RNAs in the Parasitic Nematode Family Strongyloididae. Genome Biology and Evolution. 2017;9(10):2826-43. doi: 10.1093/gbe/evx197.

4. Hunt VL, Tsai IJ, Coghlan A, Reid AJ, Holroyd N, Foth BJ, et al. The genomic basis of parasitism in the *Strongyloides* clade of nematodes. Nature genetics. 2016;48(3):299-307. Epub 2016/02/02. doi: 10.1038/ng.3495. PubMed PMID: 26829753.

5. Weinstein SB, Kuris AM. Independent origins of parasitism in Animalia. Biology letters. 2016;12(7). Epub 2016/07/21. doi: 10.1098/rsbl.2016.0324. PubMed PMID: 27436119; PubMed Central PMCID: PMCPMC4971171.

6. Crook M. The dauer hypothesis and the evolution of parasitism: 20years on and still going strong. International Journal for Parasitology. 2014;44(1):1-8. doi: https://doi.org/10.1016/j.ijpara.2013.08.004.

7. Hu PJ. Dauer. In: doi/10.1895/wormbook.1.144.1, editor. WormBook, ed The C elegans Research Community, WormBook. http://www.wormbook.org2007.

8. Wolkow CA, Hall DH. Introduction to the Dauer Larvae. WormAtlas doi:103908/wormatlasXXX. 2015.

9. Gilabert A, Curran DM, Harvey SC, Wasmuth JD. Expanding the view on the evolution of the nematode dauer signalling pathways: refinement through gene gain and pathway co-option. BMC Genomics. 2016;17(1):476. doi: 10.1186/s12864-016-2770-7.

10. Ogawa A, Streit A, Antebi A, Sommer RJ. A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. Current biology : CB. 2009;19(1):67-71. Epub 2008/12/27. doi:

10.1016/j.cub.2008.11.063. PubMed PMID: 19110431; PubMed Central PMCID: PMCPmc2712670.

11. Bento G, Ogawa A, Sommer RJ. Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. Nature. 2010;466(7305):494-7. Epub 2010/07/02. doi: 10.1038/nature09164. PubMed PMID: 20592728.

12. Antebi A, Yeh WH, Tait D, Hedgecock EM, Riddle DL. *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes & development. 2000;14(12):1512-27. Epub 2000/06/20. PubMed PMID: 10859169; PubMed Central PMCID: PMCPMC316684.

13. Lee DL. The Biology of Nematodes: CRC Press; 2002.

14. Cassada RC, Russell RL. The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology. 1975;46(2):326-42. Epub 1975/10/01. doi: 10.1016/0012-1606(75)90109-8. PubMed PMID: 1183723.

15. Dulovic A, Streit A. RNAi-mediated knockdown of daf-12 in the model parasitic nematode Strongyloides ratti. PLOS Pathogens. 2019;15(3):e1007705. doi: 10.1371/journal.ppat.1007705.

16. Wang Z, Zhou XE, Motola DL, Gao X, Suino-Powell K, Conneely A, et al. Identification of the nuclear receptor DAF-12 as a therapeutic target in parasitic nematodes. Proceedings of the National Academy of Sciences. 2009;106(23):9138-43. doi: 10.1073/pnas.0904064106.

17. Wang Z, Stoltzfus J, You Y-j, Ranjit N, Tang H, Xie Y, et al. The Nuclear Receptor DAF-12 Regulates Nutrient Metabolism and Reproductive Growth in Nematodes. PLOS Genetics. 2015;11(3):e1005027. doi: 10.1371/journal.pgen.1005027.

18. Albarqi MMY, Stoltzfus JD, Pilgrim AA, Nolan TJ, Wang Z, Kliewer SA, et al. Regulation of Life Cycle Checkpoints and Developmental Activation of Infective Larvae in *Strongyloides stercoralis* by Dafachronic Acid. PLOS Pathogens. 2016;12(1):e1005358. doi: 10.1371/journal.ppat.1005358.

19. Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, Li Y, et al. Identification of Ligands for DAF-12 that Govern Dauer Formation and

672 Reproduction in *C. elegans*. Cell. 2006;124(6):1209-23. doi:
673 https://doi.org/10.1016/j.cell.2006.01.037.
674 20. Ma G, Wang T, Korhonen PK, Stroehlein AJ, Young ND, Gasser RB. Dauer
675 signalling pathway model for Haemonchus contortus. Parasites & Vectors.
676 2019;12(1):187. doi: 10.1186/s13071-019-3419-6.
677 21. Poulin R, Randhawa HS. Evolution of parasitism along convergent lines:
678 from ecology to genomics. Parasitology. 2015;142 Suppl 1:S6-s15. Epub
679 2013/11/16. doi: 10.1017/s0031182013001674. PubMed PMID: 24229807;
680 PubMed Central PMCID: PMCPMC4413784.
681 22. Crook M, Thompson FJ, Grant WN, Viney ME. daf-7 and the development
682 of Strongyloides ratti and Parastrongyloides trichosuri. Mol Biochem Parasitol.
683 2005;139(2):213-23. Epub 2005/01/25. doi:
684 10.1016/j.molbiopara.2004.11.010. PubMed PMID: 15664656.
685 23. Gang SS, Castelletto ML, Bryant AS, Yang E, Mancuso N, Lopez JB, et al.
686 Targeted mutagenesis in a human-parasitic nematode. PLOS Pathogens.
687 2017;13(10):e1006675. doi: 10.1371/journal.ppat.1006675.
688 24. Streit A. How to become a parasite without sex chromosomes: a
689 hypothesis for the evolution of *Strongyloides* spp. and related nematodes.
690 Parasitology. 2014;141(10):1244-54. Epub 2014/05/16. doi:
691 10.1017/s003118201400064x. PubMed PMID: 24829037.
692 25. Dorris M, Viney ME, Blaxter ML. Molecular phylogenetic analysis of the
693 genus Strongyloides and related nematodes. Int J Parasitol. 2002;32(12):1507-
694 17. Epub 2002/10/24. doi: 10.1016/s0020-7519(02)00156-x. PubMed PMID:
695 12392916.
696 26. Blaxter M, Koutsovoulos G. The evolution of parasitism in Nematoda.
697 Parasitology. 2015;142 Suppl 1(Suppl 1):S26-S39. Epub 06/25. doi:
698 10.1017/S0031182014000791. PubMed PMID: 24963797.
699 27. IHGC. Comparative genomics of the major parasitic worms. Nature
700 genetics. 2019;51(1):163-74. Epub 2018/11/07. doi: 10.1038/s41588-018-
701 0262-1. PubMed PMID: 30397333; PubMed Central PMCID: PMCPMC6349046.
702 28. Fuchs G. Neue an Borken- und Rüsselkäfer gebundene Nematoden, halb
703 parasitiche und Wohnungseinmeiter1930.

29. Curcic BP, Sudhaus W, Dimitrijevic RN, Makarov SE, Tomic VT. Rhabditophanes schneideri (Rhabditida) phoretic on a cave pseudoscorpion. Journal of invertebrate pathology. 2008;99(3):254-6. Epub 2008/08/19. doi: 10.1016/j.jip.2008.07.007. PubMed PMID: 18706422.
30. Houthoofd W, Willems M, Jacobsen K, Coomans A, Borgonie G. The embryonic cell lineage of the nematode Rhabditophanes sp. The International journal of developmental biology. 2008;52(7):963-7. Epub 2008/10/29. doi: 10.1387/ijdb.072404wh. PubMed PMID: 18956326.
31. Felix MA, De Ley P, Sommer RJ, Frisse L, Nadler SA, Thomas WK, et al. Evolution of vulva development in the Cephalobina (Nematoda). Developmental biology. 2000;221(1):68-86. Epub 2000/04/25. doi: 10.1006/dbio.2000.9665. PubMed PMID: 10772792.
32. Willems M, Houthoofd W, Claeys M, Couvreur M, Van Driessche R, Adriaens D, et al. Unusual intestinal lamellae in the nematode Rhabditophanes sp. KR3021 (Nematoda: Alloinematidae). Journal of morphology. 2005;264(2):223-32. Epub 2005/03/29. doi: 10.1002/jmor.10332. PubMed PMID: 15793842.
33. Kulkarni A, Holz A, Rodelsperger C, Harbecke D, Streit A. Differential chromatin amplification and chromosome complements in the germline of Strongyloididae (Nematoda). Chromosoma. 2016;125(1):125-36. Epub 2015/07/25. doi: 10.1007/s00412-015-0532-y. PubMed PMID: 26205504.
34. Karp X. Working with dauer larvae. WormBook : the online review of C elegans biology. 2018;2018:1-19. doi: 10.1895/wormbook.1.180.1. PubMed PMID: 27417559.
35. Viney M. The genomic basis of nematode parasitism. Briefings in functional genomics. 2018;17(1):8-14. Epub 2017/05/05. doi: 10.1093/bfgp/elx010. PubMed PMID: 28472353; PubMed Central PMCID: PMCPMC5886223.
36. Ma G, Wang T, Korhonen PK, Young ND, Nie S, Ang C-S, et al. Dafachronic acid promotes larval development in Haemonchus contortus by modulating dauer signalling and lipid metabolism. PLOS Pathogens. 2019;15(7):e1007960. doi: 10.1371/journal.ppat.1007960.

736 37. Pires-daSilva A. Pristionchus pacificus protocols. WormBook, ed The C
737 elegans Research Community, Wormbook, doi/101895/wormbook11142,
738 http://wwwwormbookorg. 2013.
739 38. Shaham S. Methods in Cell Biology (January 02, 2006). WormBook, ed The
740 C elegans Research Community, Wormbook, doi/101895/wormbook1491,
741 http://wwwwormbookorg. 2006.
742 39. Dalzell JJ, McVeigh P, Warnock ND, Mitreva M, Bird DM, Abad P, et al. RNAi
743 Effector Diversity in Nematodes. PLOS Neglected Tropical Diseases.
744 2011;5(6):e1176. doi: 10.1371/journal.pntd.0001176.
745

Fig 1

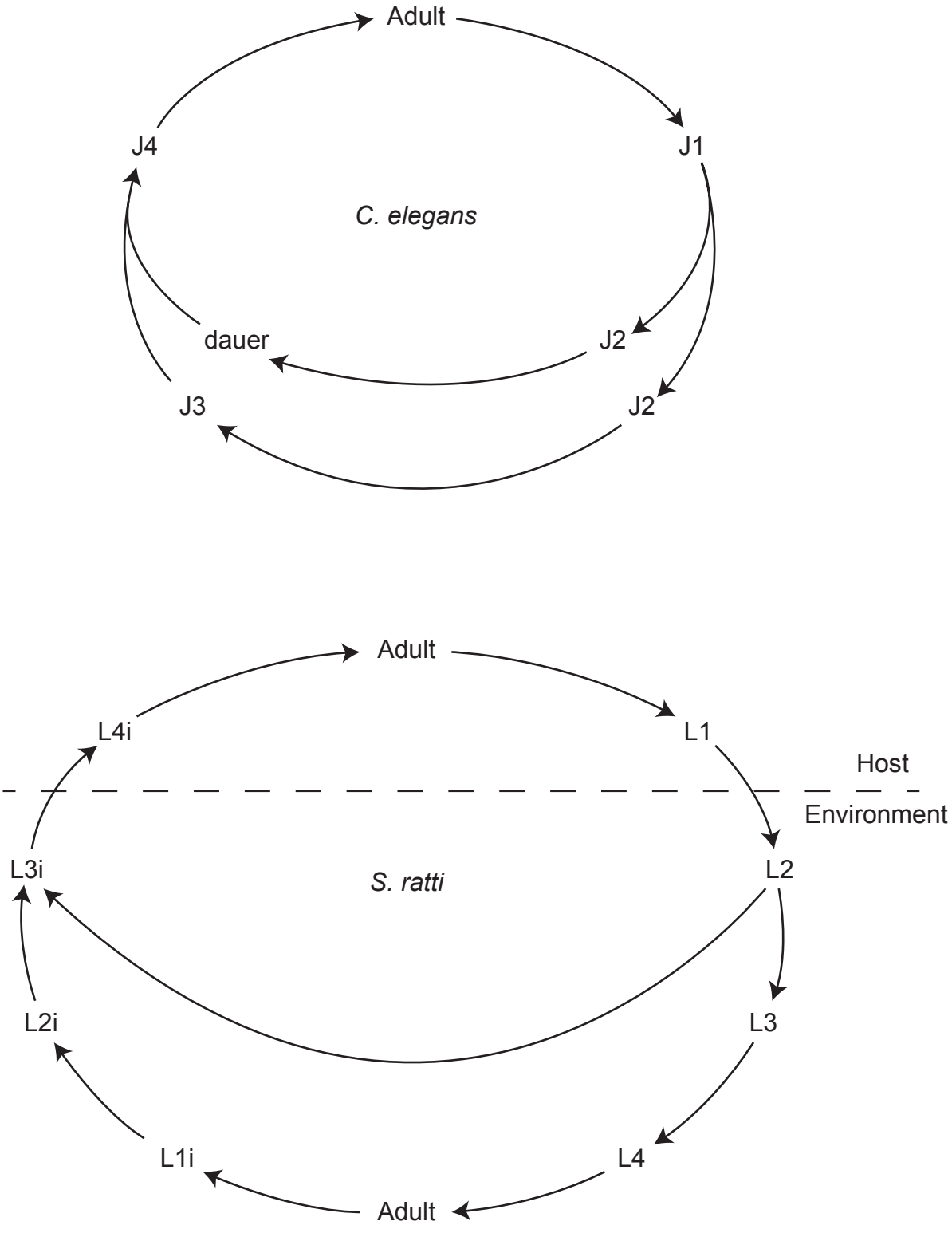

Adult
J1
J2
J2
J3
dauer
J4
C. elegans
Adult
L1
L2
L3
L4
Adult
L1i
L2i
L3i
L4i
Host
Environment
S. ratti

Fig 2

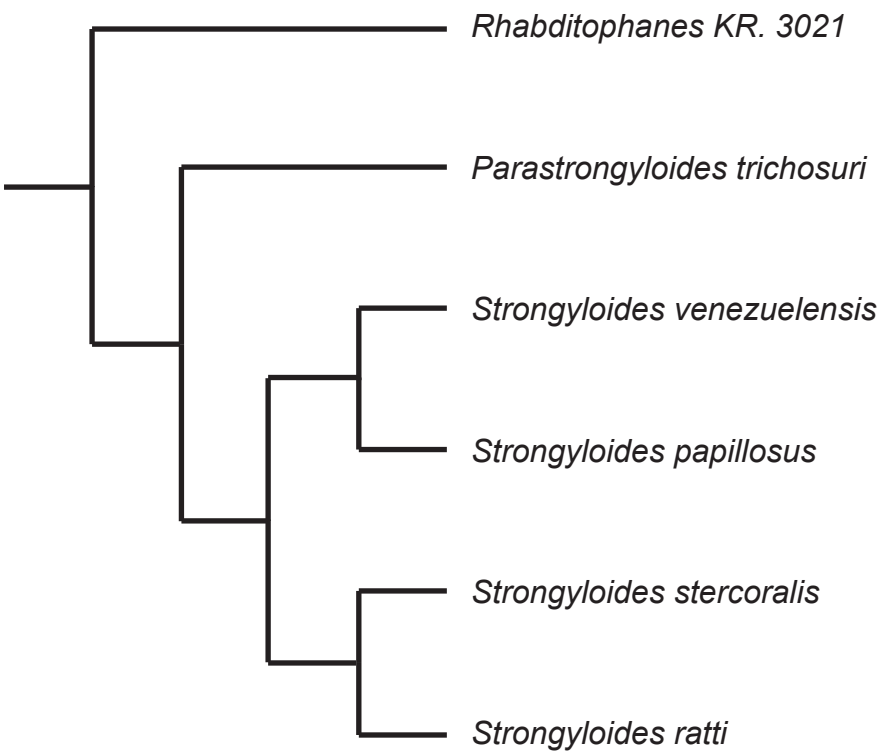

Rhabditophanes KR. 3021
Parastrongyloides trichosuri
Strongyloides venezuelensis
Strongyloides papillosus
Strongyloides stercoralis
Strongyloides ratti

Fig 3

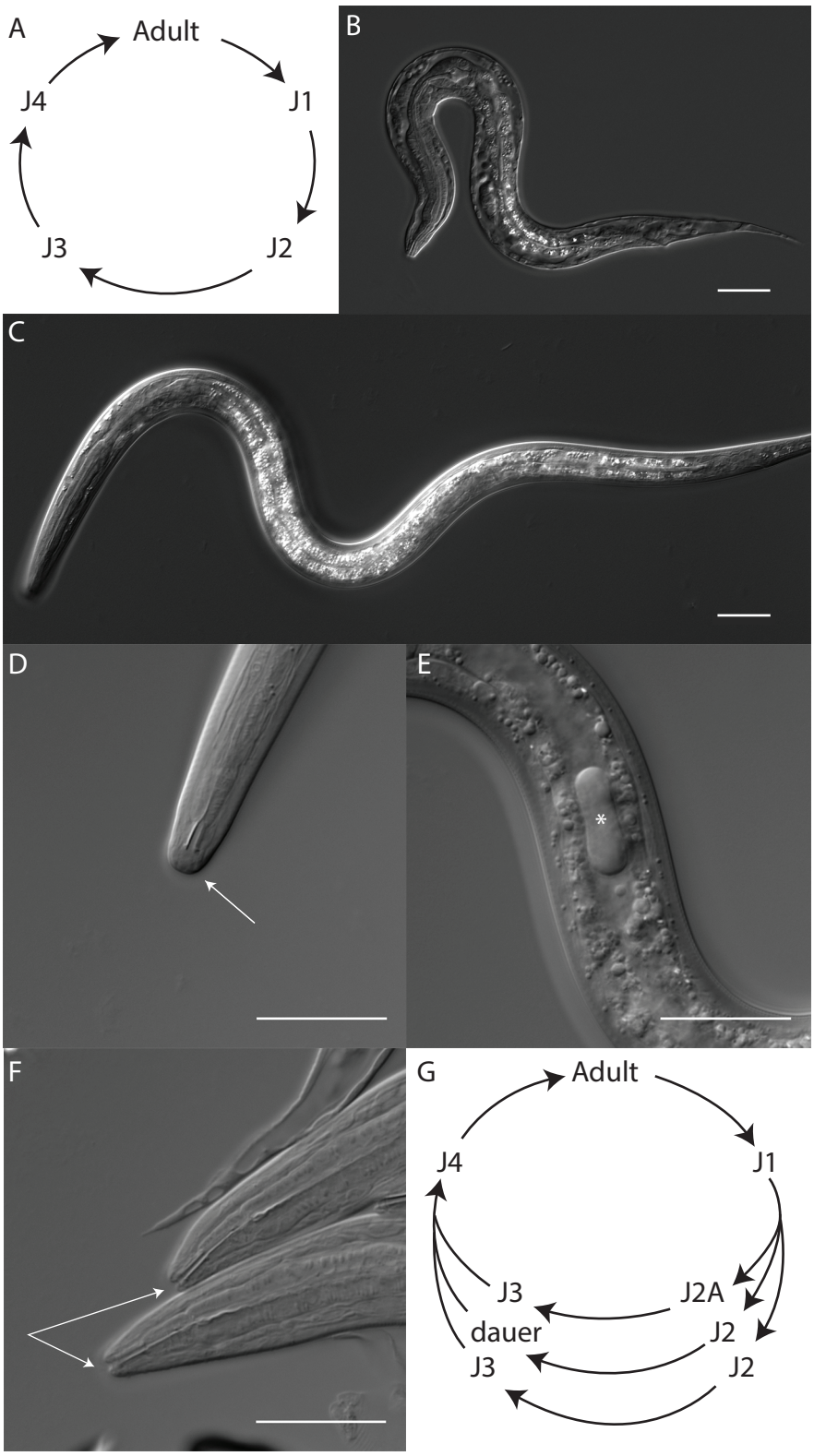
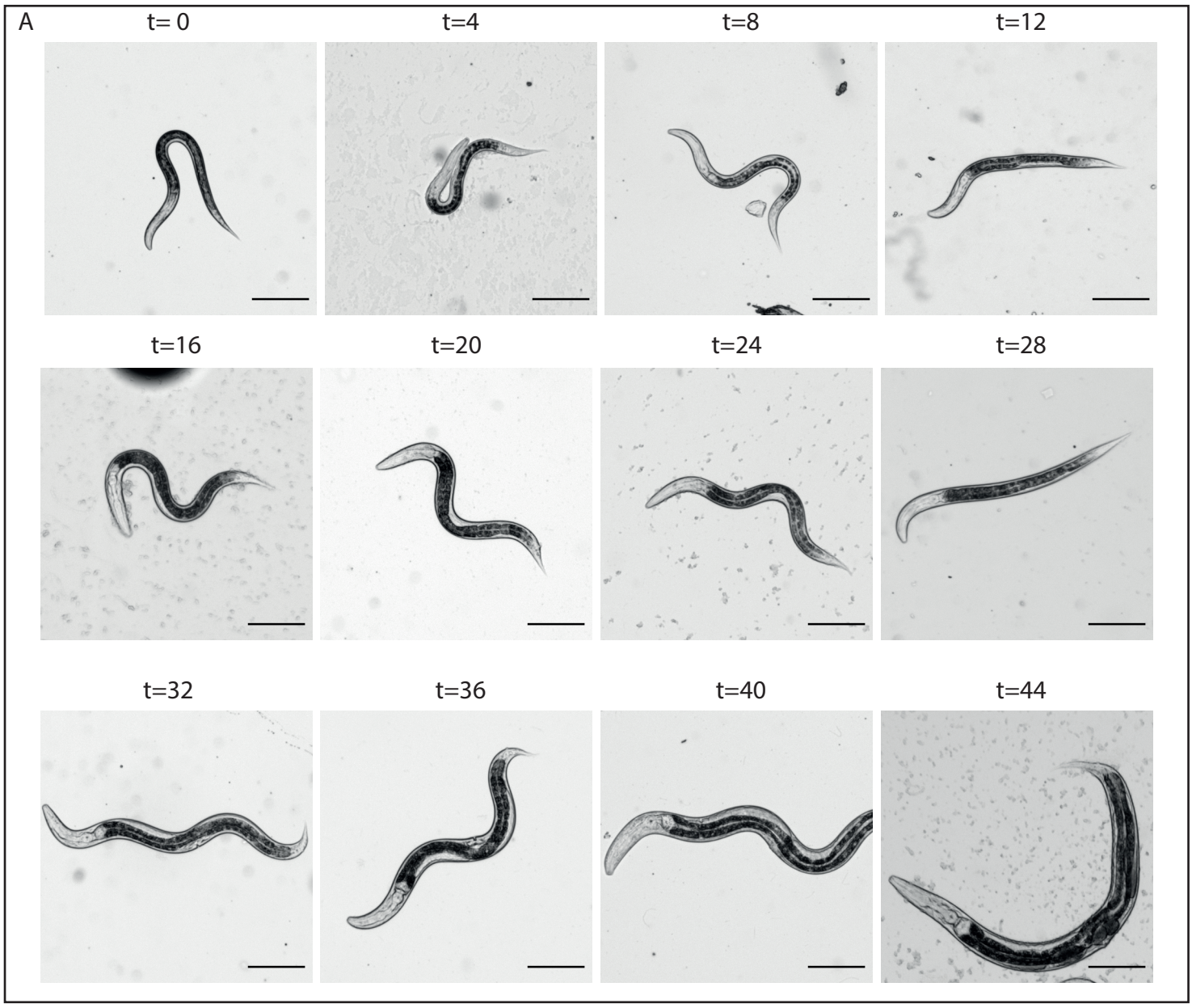
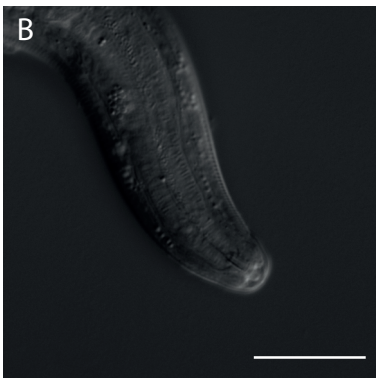
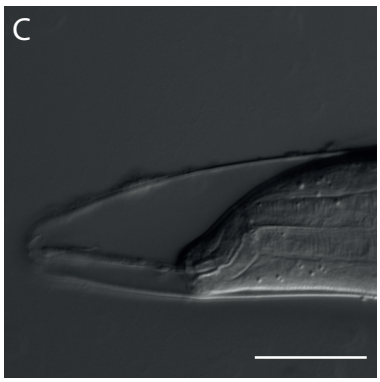


Fig 4

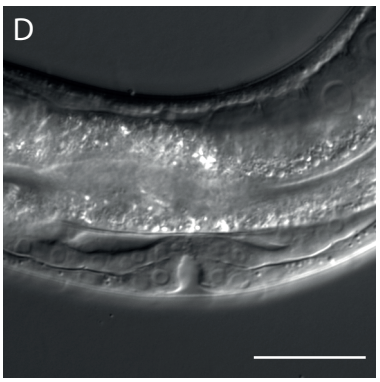




Dauer to J4 molt



J4 to Adult molt - mouth view



J4 to Adult molt - vulva view

Fig 5

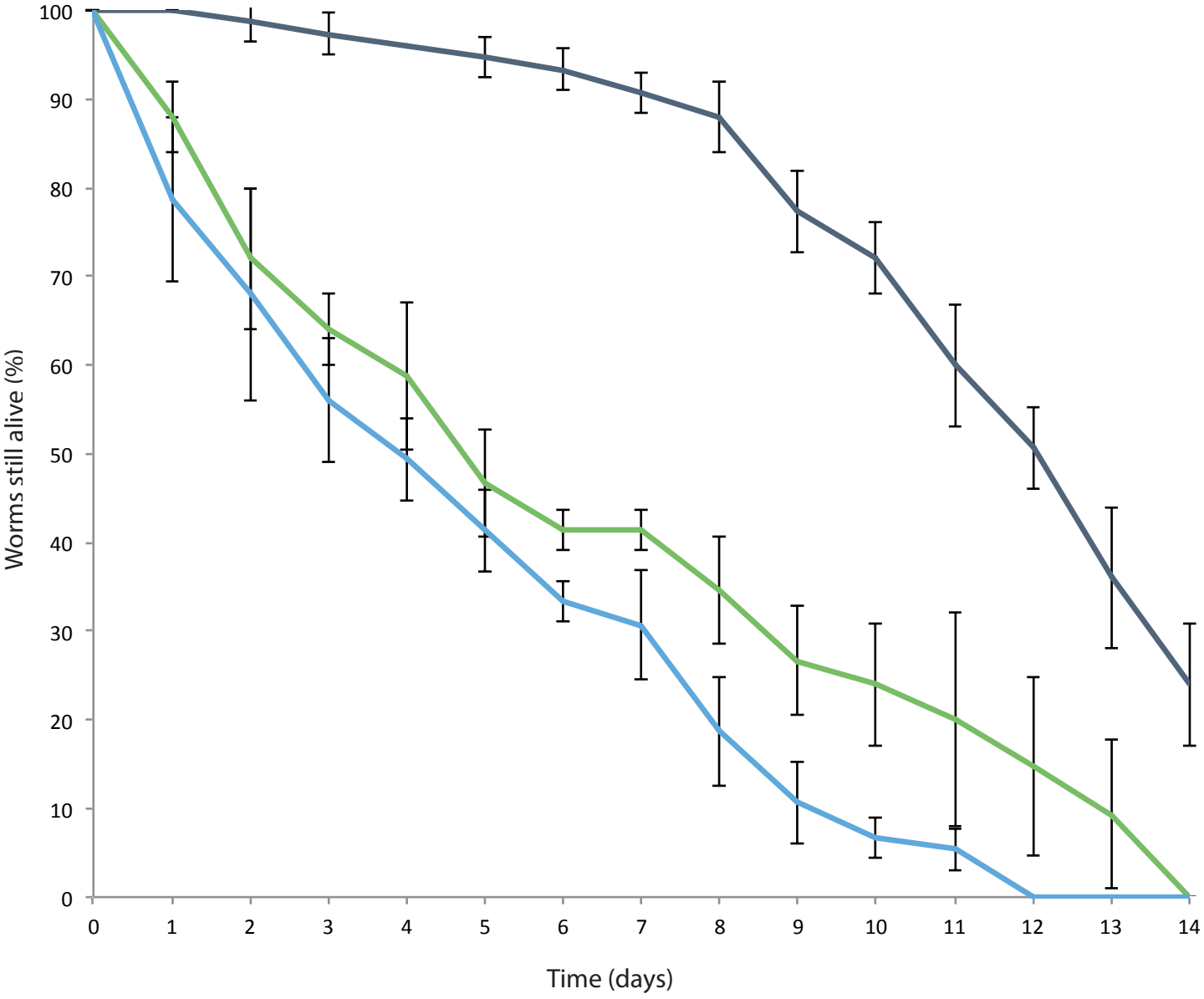

100
90
80
70
60
50
40
30
20
10
0
Worms still alive (%)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

Fig 6

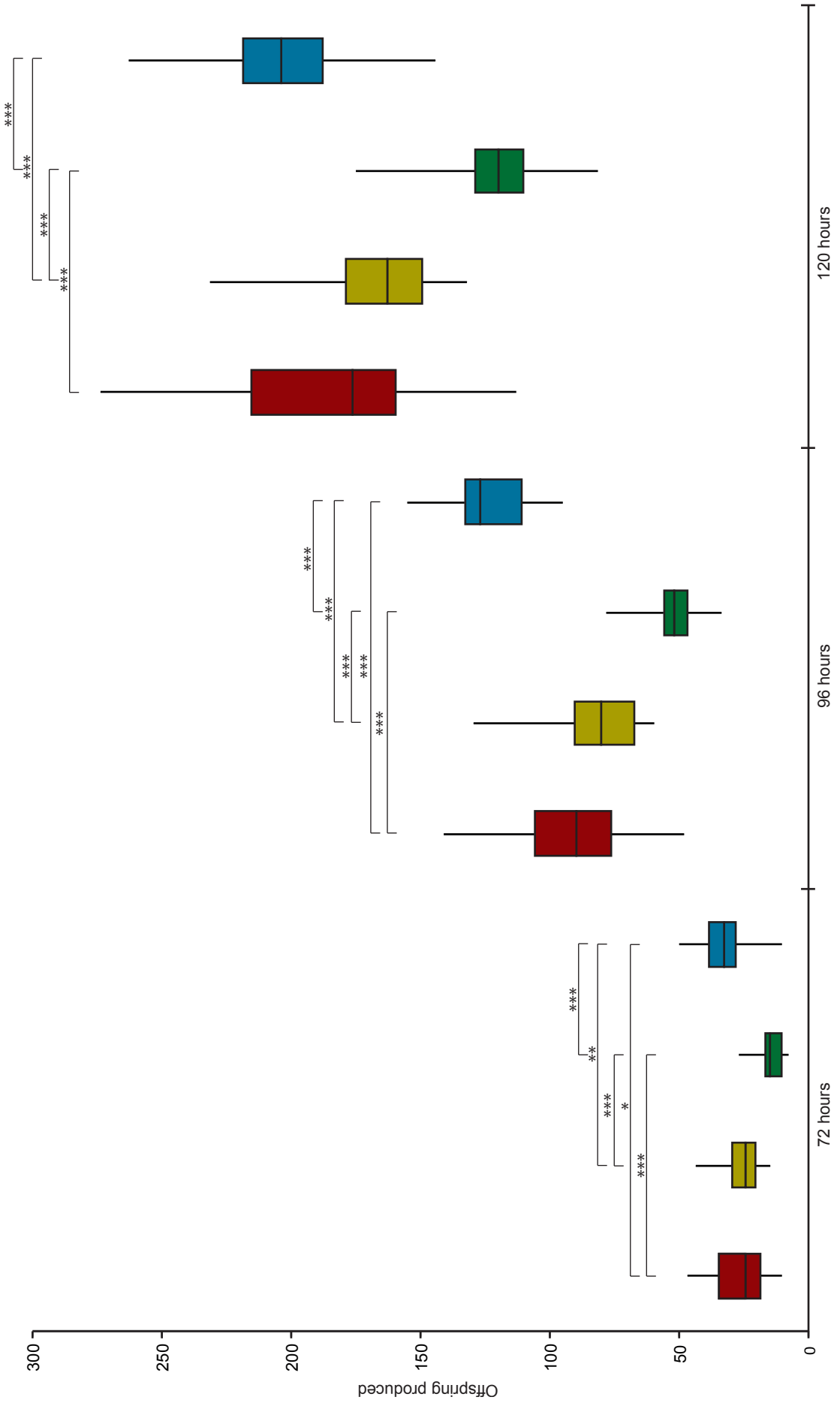
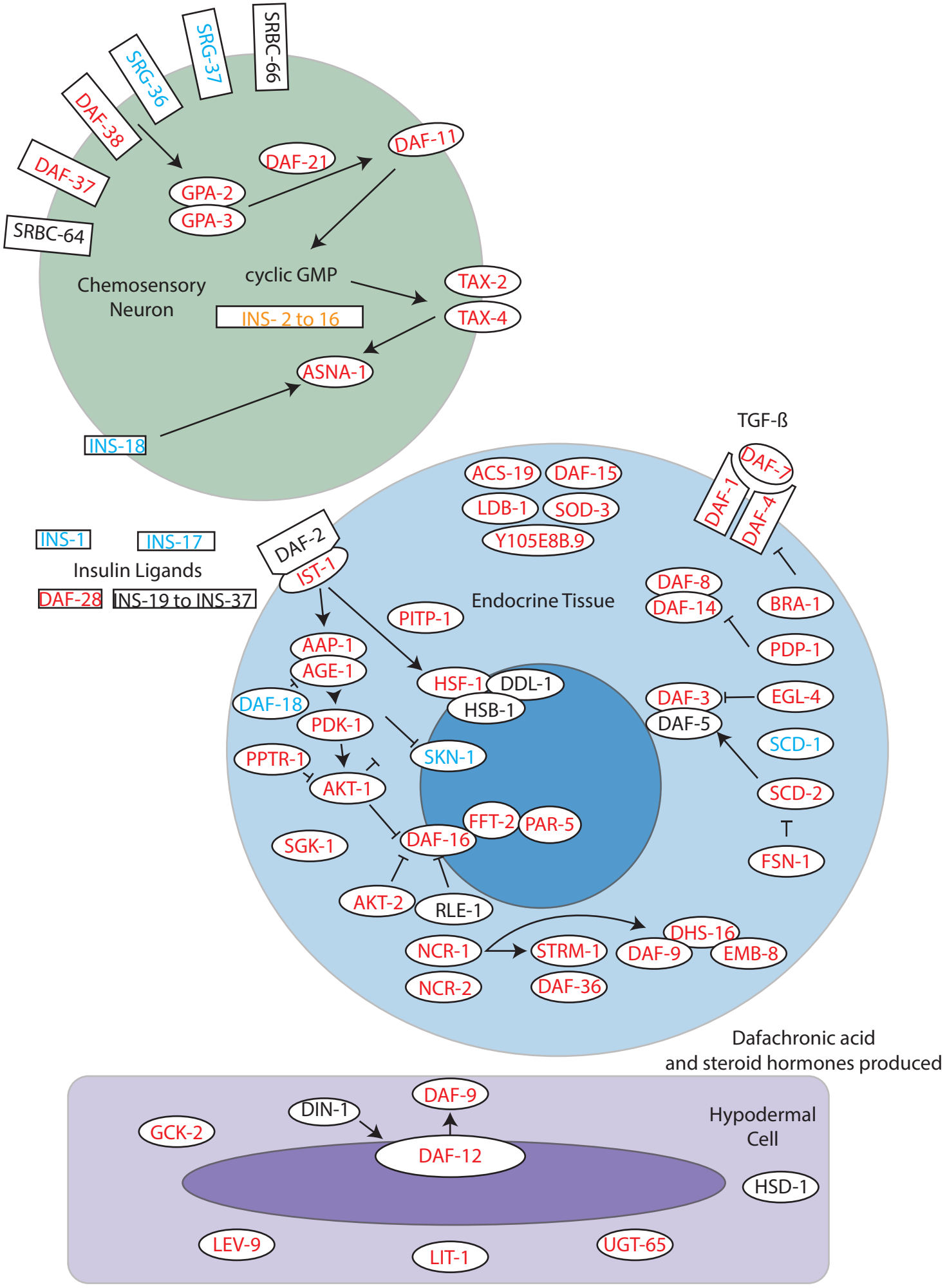

Offspring produced
300
250
200
150
100
50
0
72 hours
96 hours
120 hours
***
**
***
*
***
***
***
***
***
***
***
***
***
***
***
***

Fig 7

Pathway	Gene	RSKR	SRAE	SPAL	Pathway	Gene	RSKR	SRAE	SPAL	Pathway	Gene	RSKR	SRAE	SPAL
cGMP	*daf-11*	■	■	■	Insulin Signaling	*daf-18*	■			Insulin Signaling	*ins-29*			
	daf-21	■	■	■		*daf-28*	■		■		*ins-30*			
	daf-37	■	■	■		*ddl-1*					*ins-31*			
	daf-38	■	■	■		*ftt-2*	■	■	■		*ins-32*			
	gpa-2	■	■	■		*hsb-1*					*ins-33*			
	gpa-3	■	■	■		*hsf-1*	■	■	■		*ins-34*			
	srbc-64					*ins-1*	■				*ins-35*			
	srbc-66					*ins-2*					*ins-36*			
	srg-36	■				*ins-3*	■	■			*ins-37*			
	srg-37	■				*ins-4*	■				*ist-1*	■	■	■
	tax-2	■	■	■		*ins-5*					*ldb-1*	■	■	■
	tax-4	■	■	■		*ins-6*	■		■		*par-5*	■	■	■
TGF-ß	*bra-1*	■	■	■		*ins-7*	■	■	■		*pdk-1*	■	■	■
	daf-1	■	■	■		*ins-8*	■		■		*pitp-1*	■	■	■
	daf-3	■	■	■		*ins-9*					*pptr-1*	■	■	■
	daf-4	■	■	■		*ins-10*					*rle-1*			
	daf-5					*ins-11*					*sgk-1*	■	■	■
	daf-7	■	■	■		*ins-12*					*skn-1*	■		
	daf-8	■	■	■		*ins-13*					*sod-3*	■	■	■
	daf-14	■	■	■		*ins-14*					*Y105E8B.9*	■	■	■
	egl-4	■	■	■		*ins-15*				Steroid Hormone	*daf-9*	■	■	■
	fsn-1	■	■	■		*ins-16*					*daf-12*	■	■	■
	pdp-1	■	■	■		*ins-17*	■				*daf-36*	■	■	■
	scd-1	■				*ins-18*	■				*dhs-16*	■	■	■
	scd-2	■	■	■		*ins-19*					*din-1*			
Insulin Signaling	*aap-1*	■	■	■		*ins-20*					*emb-8*	■	■	■
	acs-19	■	■	■		*ins-21*					*gck-2*	■	■	■
	age-1	■	■	■		*ins-22*					*hsd-1*			
	akt-1	■	■	■		*ins-23*					*lev-9*	■	■	■
	akt-2	■	■	■		*ins-24*					*lit-1*	■	■	■
	asna-1	■	■	■		*ins-25*					*ncr-1*	■	■	■
	daf-2					*ins-26*					*ncr-2*	■	■	■
	daf-15	■	■	■		*ins-27*					*strm-1*	■	■	■
	daf-16	■	■	■		*ins-28*					*ugt-65*	■	■	■

Fig 8


SRBC-66
SRG-37
SRG-36
DAF-38
DAF-37
SRBC-64
DAF-21
DAF-11
GPA-2
GPA-3
Chemosensory Neuron
cyclic GMP
INS- 2 to 16
TAX-2
TAX-4
ASNA-1
INS-18
INS-1
INS-17
Insulin Ligands
DAF-28
INS-19 to INS-37
TGF-ß
DAF-7
DAF-1
DAF-4
ACS-19
DAF-15
LDB-1
SOD-3
Y105E8B.9
DAF-2
IST-1
Endocrine Tissue
DAF-8
DAF-14
BRA-1
PDP-1
PITP-1
AAP-1
AGE-1
HSF-1
DDL-1
HSB-1
DAF-18
PDK-1
SKN-1
DAF-3
EGL-4
DAF-5
SCD-1
PPTR-1
AKT-1
SCD-2
FFT-2
PAR-5
SGK-1
DAF-16
FSN-1
AKT-2
RLE-1
DHS-16
NCR-1
STRM-1
DAF-9
EMB-8
NCR-2
DAF-36
Dafachronic acid
and steroid hormones produced
DAF-9
DIN-1
GCK-2
DAF-12
Hypodermal Cell
HSD-1
LEV-9
LIT-1
UGT-65

Supplementary Tables

Rhabditophanes diutinus Basic Species Description

Adults: Cylindrical body shape, 705μm long and 54μm wide. Lip regions consists of four sectors, each with a labial sensillum. Narrow buccal cavity (4μm), with large pharynx consisting of a long narrow cylindrical procorpus (46 μm long, 10μm wide), a small rounded metacorpus (24μm long, 17μm wide), a long thin cylindrical isthmus (32μm long, 10μm wide) and a large circular well-rounded posterior bulb (26μm by 25μm). Nerve ring sits over isthmus. Grinder appears present within the posterior bulb. Excretory pore present in the isthmus region. Intestine runs length of body, 26-29μm wide, from directly after the pharynx to the rectum. The intestine is not fixed to the body wall and is wrapped around the gonads. Well developed sphincter following gonadal loop. Rectum is short and wide, with anus a raised opening in the form of a vertical slit in the cuticle. Vulva located at mid body, with a horizontal slit. Gonads are didelphic and wrap around the intestine, posterior arm is as a result normally hidden by the intestine. The gonad arms extend past the vulva in both directions. Germ cells are arranged in giant nuclei which are easily observable under DIC (10-12μm in size). Following the gonadal loop, undeveloped oocytes are present. These oocytes have a shiny appearance and are rich in cytoplasmic material. Whilst it appears that there is a spermatotheca, these cells do not have nuclei. Maturing oocytes pass through them, after which they begin developing into embryos. Embryos are less common in other species and it is rare to see ever more than 2 per gonad arm in development. The exact development stage of the embryo when laid appears inconsistent. Embryos are 55.04μm when laid.

Dauer Larvae: Cylindrical body shape, 403μm long with a large amount of radial constriction (18μm wide at midpoint). Dauers are marked by the presence of a large buccal and intestinal plug consisting of multiple parts. Buccal cavity is narrow and restricted, with a large buccal plug. Further plugs are found in the upper third of the intestine, mid intestine, and lower third of intestine. These plugs appear shiny when viewed under DIC. Gonadal development is limited to a

small region, consisting of 4 to 8 nuclei, within a small smooth structure. Gut lumen is large and full of bacteria. No vulval development. Cuticle is thicker and striated compared to adult stages.

J2A Larvae: Cylindrical body shape, 262µm in length, proportionally appears as J2 worm. Mouth consists of 33% of total body length. Buccal cavity is narrow and open, pharynx fully developed. Intestine appears empty, lumen wall is thinner than other stages. Gonad consists of two progenitor cells. All other intracellular organelles appear endocytosed dependent on how long the worm has been in this stage for. Tail is short and triangular. Cuticle is striated. When in this stage for a prolonged period of time, the only identifiable features are the mouth, intestine and germline.

Supplementary Table 1

Category	Adult	Dauer	J2A
Body length	658 – 759 (704.6)	339 – 442 (402.8)	249,84 – 292,31 (262.00)
Body width at oesophagus	33.42 – 43.86 (40.00)	15.96 – 17-61 (16.70)	16.14 – 17-95 (17.09)
Width of buccal cavity	2.94 – 4.10 (3.58)	0,96 – 1.16 (1.04)	0.58 – 1.09 (0.84)
Width of terminal bulb	16.52 – 22-49 (20.38)	7.96 – 8.96 (8.46)	8.69 – 11-05 (10,13)
Oesophagus length	95.00 – 145 (118.90)	65.69 – 79.83 (74.91)	78.91 – 92.79 (85.54)
Oesophagus of total (%)	16.87%	18.60	32.65
Intestinal lumen width	4.76 – 5.86 (5.16)	2.99 – 3.38 (3.13)	1.08 – 2.73 (1.90)
Intestinal wall width	9.08 – 11-53 (10.30)	3.84 – 4.54 (4.16)	2.71 – 4.14 (3.41)
Ratio of Intestinal lumen to intestinal wall	0.50	0.75	0.56
Vulva/Germ cells from anterior	354.24 – 404.85 (374.00)	186.42 – 226.00 (209.39)	128.67 – 164,12 (145,10)
Vulva/Germ cells from anterior as % of body length	53.02	51.98	55.38
Width at vulva	48.62 – 62.18 (54.47)	16.62 – 18.84 (17.64)	12.11 – 15.33 (14.51)
Length of gonad	177,98 – 256-72 (216.08)	27.48 – 30.04 (29.07)	11.03 – 13.19 (12.05)
Tail length	75.99 – 93.38 (86.17)	32.59 – 34.87 (33.97)	25.91 – 35.52 (29.81)

Measurements of key morphological characteristics between Adult, Dauer and J2A worms. As J2A and dauers do not have a vulva, the germ cell block is used in its place. All measurements are in µm, mean shown in brackets.

Supplementary Table 2

Genes present within the cyclic GMP pathway of dauer development in *Rhabditophanes diutinus (RSKR), Strongyloides ratti (SRAE)* and *Strongyloides papillosus (SPAL).*

C elegans Gene	WormBaseID	Function	RSKR Gene	SRAE Gene	SPAL Gene
Ce-daf-11	WBGene00000907	Receptor-type guanylate cyclase	RSKR_0000013600	SRAE_2000430600	SPAL_0001362700
			RSKR_0000021800	SRAE_0000007100	
			RSKR_0000682500	SRAE_X000020900	
			RSKR_0000864300		
			RSKR_0000865900		
			RSKR_0000983300		
			RKSR_0000988100		
			RSKR_0001069500		
			RSKR_0001110100		
Ce-daf-21	WBGene00000915	Heat shock protein 90	RSKR_0000116900	SRAE_1000116300	SPAL_0000899600
			RSKR_0000134300	SRAE_0000032700	SPAL_0001592000
			RSKR_0000745600		
			RSKR_0001154400		
Ce-daf-37	WBGene00016246	G-protein coupled receptor daf-37	RSKR_0001026800	SRAE_1000218800	SPAL_0001213100
			RSKR_0001127100		
Ce-daf-38	WBGene00013642	G-protein coupled receptor daf-38	RSKR_0000265100	SRAE_2000284200	SPAL_0000888700
			RSKR_0001177100		
Ce-gpa-3	WBGene00001665	Guanine nucleotide-binding protein alpha-3 subunit	RSKR_0000116000	SRAE_1000244900	SPAL_0000749500
			RSKR_0000774600	SRAE_1000354700	SPAL_0000488700
			RSKR_0000502500	SRAE_2000369300	
Ce-tax-2	WBGene00006525	Cyclic nucleotide-gated cation channel subunit	RSKR_0000484800	SRAE_1000291000	SPAL_0001119900
			RSKR_0000486900		
			RSKR_0000128900		
Ce-tax-4	WBGene00006526	Cyclic nucleotide-gated cation channel subunit	RSKR_0001107200	SRAE_2000234000	SPAL_0001371800
Ce-gpa-2	WBGene00001664	Guanine nucleotide-binding protein alpha-2 subunit	RSKR_0000502500	SRAE_2000369300	SPAL_0000465200
			RSKR_0000116000		
			RSKR_0000774600		
Ce-srg-36	WBGene00005193	G-protein coupled receptor	RSKR_0000234766		
Ce-srg-37	WBGene00005194	G-protein coupled receptor	RSKR_0000877333		

Supplementary Table 3

Genes present within the TGF-ß pathway of dauer development in *Rhabditophanes diutinus (RSKR), Strongyloides ratti (SRAE)* and *Strongyloides papillosus (SPAL).*

C elegans Gene	WormBaseID	Function	RSKR Gene	SRAE Gene	SPAL Gene
Ce-daf-1	WBGene00000897	Cell surface receptor	RSKR_0000584300	SRAE_X000145000	SPAL_0000386100
Ce-daf-14	WBGene00000910	Smad related ptotein	RSKR_0000082500	SRAE_1000265800	SPAL_0001290200
			RSKR_0000926700	SRAE_1000296900	SPAL_0000266700
			RSKR_0000114900		
Ce-daf-3	WBGene00000899	Co-smad protein	RSKR_0000082500	SRAE_2000081300	SPAL_0001099400
			RSKR_0000926700	SRAE_2000433800	
			RSKR_0000114900		
Ce-daf-4	WBGene00000900	Cell surface receptor daf-4	RSKR_0000928900	SRAE_2000055000	SPAL_0000609900
Ce-daf-7	WBGene00000903	Dauer larva development regulatory growth factor daf-7	RSKR_0000738300	SRAE_2000440900	SPAL_0001430900
				SRAE_X000046300	SPAL_0000060000
				SRAE_2000480100	SPAL_0001086000
				SRAE_1000133700	
Ce-daf-8	WBGene00000904	Smad protein	RSKR_0000926700	SRAE_2000151100	SPAL_0000096100
			RSKR_0000082500		
			RSKR_0000114900		
Ce-egl-4	WBGene00001173	Cyclic GMP-dependent protein kinase	RSKR_0000752400	SRAE_X000181200	SPAL_0000923200
					SPAL_0001514000
Ce-fsn-1	WBGene00001499	Fbox protein	RSKR_0000226700	SRAE_2000092400	SPAL_0000785600
Ce-pdp-1	WBGene00022832	Pyruvate dehyrogenase phosphatase catalytic subunit	RSKR_0000271100	SRAE_2000335300	SPAL_0000220200
Ce-scd-1	WBGene00004739	ALK tyrosine kinase receptor homolog scd-1	RSKR_0000404900		
Ce-scd-2	WBGene00004740	ALK tyrosine kinase receptor homolog scd-2	RSKR_0000333200	SRAE_1000072600	SPAL_0000741500
Ce-bra-1	WBGene00000262	ortholog of human ZMYND11 (zinc finger MYND-type containing 11) and ZMYND8 (zinc finger MYND-type containing 8)	RSKR_0000926900	SRAE_1000266000	SPAL_0000266900

Supplementary Table 4

Genes present within the Insulin signaling pathway of dauer development in *Rhabditophanes diutinus (RSKR), Strongyloides ratti (SRAE)* and *Strongyloides papillosus (SPAL).*

C elegans Gene	WormBaseID	Function	RSKR Gene	SRAE Gene	SPAL Gene
Ce-aap-1	WBGene00000001	phosphoinositide kinase AdAPter subunit	RSKR_0000346700	SRAE_2000145000	SPAL_0001698500
Ce-acs-19	WBGene00007969	Acetyl-coenzyme A synthetase	RSKR_0000070600	SRAE_2000037500	SPAL_0000969700
Ce-age-1	WBGene00000090	Phosphatidylinositol 3-kinase age-1	RSKR_0000800700	SRAE_X000092700	SPAL_0001610900
Ce-akt-1	WBGene00000102	Serine/threonine-protein kinase akt-1	RSKR_0000974300	SRAE_2000477900	SPAL_0000605700
Ce-asna-1	WBGene00014025	ATPase asna-1	RSKR_0000122500	SRAE_2000240000	SPAL_0001201500
Ce-daf-15	WBGene00000911	Phosphoinositide 3-kinase (PI3K) p50/p55 regulatory subunit	RSKR_0000372200	SRAE_1000150700	SPAL_0001577200
Ce-daf-16	WBGene00000912	Forkhead box protein O	RSKR_0000006600	SRAE_1000273700	SPAL_0000140400
Ce-daf-18	WBGene00000913	Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase	RSKR_0000830000		
Ce-daf-2	WBGene00000898	Receptor protein-tyrosine kinase	RSKR_0000957200		SPAL_0001756600
Ce-ftt-2	WBGene00001502	14-3-3-like protein 2	RSKR_0001129200	SRAE_2000364600	SPAL_0000470300
			RSKR_0001127600	SRAE_2000264800	SPAL_0000470500
Ce-hsf-1	WBGene00002004	Heat shock factor protein 1	RKSR_0000052100	SRAE_1000030300	SPAL_0000387400
				SRAE_2000113800	SPAL_0000387600
				SRAE_1000030600	SPAL_0001635100
Ce-ins-1	WBGene00002084	Insulin-like peptide 1	RSKR_0000746100		
Ce-ins-17	WBGene00002100	Insulin-like peptide 17	RSKR_0000984000		
Ce-ins-18	WBGene00002101	Insulin-like peptide 18	RSKR_0000984000		
Ce-ist-1	WBGene00002163	pleckstrin homology (PH) and phosphotyrosine binding (PTB) domain-containing insulin receptor substrate (IRS) homolog that negatively	RSKR_0001182300	SRAE_2000381500	SPAL_0000419000
Ce-ldb-1	WBGene00002261	Nuclear LIM binding protein	RSKR_0000184200	SRAE_2000338200	SPAL_0001714600
				SRAE_X000031000	SPAL_0001608600
					SPAL_0000223100
Ce-pdk-1	WBGene00003965	3-phosphoinositide-dependent protein kinase 1	RSKR_0000865500	SRAE_X000167000	SPAL_0000356000
Ce-pitp-1	WBGene00010813	phosphatidylinositol transfer protein	RSKR_0000219200	SRAE_2000097600	SPAL_0000770600
Ce-pptr-1	WBGene00012348	Serine/threonine-protein phosphatase 2A regulatory subunit pptr-1	RSKR_0000669100	SRAE_1000015200	SPAL_0000723700
Ce-sgk-1	WBGene00004789	Serine/threonine-protein kinase sgk-1	RSKR_0000826200	SRAE_X000137400	SPAL_0001386800
			RSKR_0000183700		
Ce-skn-1	WBGene00004804	bZip transcription factor	RSKR_0000815900		
Ce-sod-3	WBGene00004932	iron/manganese superoxide dismutase	RSKR_0000618100	SRAE_1000313400	SPAL_0001269900
			RSKR_0000015300		
Ce-Y105E8B.9	WBGene00013693	Glutathione transferase	RSKR_0000004400	SRAE_2000082500	SPAL_0000776300
Ce-akt-2	WBGene00000103	Serine/threonine-protein kinase akt-2	RSKR_0000974300	SRAE_2000477900	SPAL_0000605700
Ce-ins-3	WBGene00002086	insulin-like peptide	RSKR_0000746100	SRAE_1000196300	
Ce-ins-4	WBGene00002087	insulin-like peptide	RSKR_0000790350		
Ce-ins-6	WBGene00002089	insulin-like peptide	RSKR_0000746100		SPAL_0000402900
			RKSR_0000790350		
Ce-ins-7	WBGene00002090	insulin-like peptide	RSKR_0000790350	SRAE_X000099000	SPAL_0000402900
					SPAL_0001579700
Ce-ins-8	WBGene00002091	insulin-like peptide	RSKR_0000790350		SPAL_0000402900
Ce-par-5	WBGene00003920	14-3-3 proteins	RSKR_0001127600	SRAE_2000364600	SPAL_0000470300
			RSKR_0001129200	SRAE_2000364800	SPAL_0000470500
Ce-ins-2	WBGene00002085	Insulin-like peptide			SPAL_0001579700

Supplementary Table 5

Genes present within the steroid hormone signaling pathway of dauer development in *Rhabditophanes diutinus (RSKR), Strongyloides ratti (SRAE)* and *Strongyloides papillosus (SPAL).*

C elegans Gene	WormBaseID	Function	RSKR Gene	SRAE Gene	SPAL Gene
Ce-daf-12	WBGene00000908	Nuclear hormone receptor family member daf-12	RSKR_0001015700	SRAE_0000032100	SPAL_0001591300
Ce-daf-36	WBGene00007536	Cholesterol 7-desaturase	RSKR_0000775200	SRAE_2000135900	SPAL_0000182600
			RSKR_0000917000		
Ce-daf-9	WBGene00000905	Cytochrome P450	RSKR_0000045600	SRAE_1000164700	SPAL_0000650300
			RSKR_0000885200	SRAE_X000032600	SPAL_0001193000
			RSKR_0000486300	SRAE_1000133100	SPAL_0001086700
			RSKR_0000389700		SPAL_0001528500
			RSKR_0000117200		SPAL_0001333100
			RSKR_0000162400		
			RSKR_0000324700		
			RSKR_0000203700		
			RSKR_0000460900		
			RSKR_0000466000		
			RSKR_0000485800		
			RSKR_0000524900		
			RSKR_0000552100		
			RSKR_0000616800		
			RSKR_0000693700		
			RSKR_0000714100		
Ce-dhs-16	WBGene00000979	3 beta-hydroxysteroid dehydrogenase dhs-16	RSKR_0000971900	SRAE_1000165700	SPAL_0000651500
			RSKR_0000076600		
			RSKR_0000071400		
Ce-emb-8	WBGene00001262	NADPH--cytochrome P450 reductase	RSKR_0000093300	SRAE_2000243000	SPAL_0000907400
Ce-gck-2	WBGene00022603	Mitogen-activated protein kinase kinase kinase kinase	RSKR_0000894700	SRAE_1000138500	SPAL_0000753000
Ce-lev-9	WBGene00002976	Sushi domain containing protein	RSKR_0000889400	SRAE_2000488800	SPAL_0001157100
Ce-lit-1	WBGene00003048	Serine/threonine kinase NLK	RSKR_0000605600	SRAE_2000104300	SPAL_0000218200
			RSKR_0000206900	SRAE_2000333400	SPAL_0000999600
Ce-ncr-1	WBGene00003561	Niemann-Pick C1 protein homolog 1	RSKR_0001181600	SRAE_1000068300	SPAL_0000479300
Ce-strm-1	WBGene00019198	Sterol a-ring methylase	RSKR_0000954600	SRAE_2000165300	SPAL_0001624200
Ce-ugt-65	WBGene00008583	UDP-glucuronosyltransferase	RSKR_0000386000	SRAE_1000191300	SPAL_0000464600
			RSKR_0000832500	SRAE_2000236200	SPAL_0001512500
				SRAE_2000498200	SPAL_0001048400
				SRAE_2000498300	SPAL_0000620200
				SRAE_X000170500	SPAL_0000357900
				SRAE_2000325500	SPAL_0001412800
					SPAL_0001740400
Ce-ncr-2	WBGene00003562	Niemann-Pick C1 protein homolog 2	RSKR_0001181600	SRAE_1000068300	SPAL_0000479300
					SPAL_0000027700

References

1. Lee DL. The Biology of Nematodes: CRC Press; 2002.

2. Perry RN, Wharton DA. Molecular and physiological basis of nematode survival: CAB International; 2011.

3. Blaxter M, Denver DR. The worm in the world and the world in the worm. BMC biology. 2012;10:57. Epub 2012/06/27. doi: 10.1186/1741-7007-10-57. PubMed PMID: 22731915; PubMed Central PMCID: PMCPMC3382423.

4. Zhang ZQ. Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness (Addenda 2013). Zootaxa. 2013;3703:1-82. Epub 2013/01/01. doi: 10.11646/zootaxa.3703.1.1. PubMed PMID: 26146682.

5. Lambshead PJD. Recent developments in marine benthic biodiversity research. Oceanis. 1993;19(6):5-24.

6. Pires-daSilva A. Evolution of the control of sexual identity in nematodes. Seminars in Cell & Developmental Biology. 2007;18(3):362-70. doi: https://doi.org/10.1016/j.semcdb.2006.11.014.

7. Consortium TCeG. Genome sequence of the nematode C. elegans: a platform for investigating biology. Science (New York, NY). 1998;282(5396):2012-8. Epub 1998/12/16. doi: 10.1126/science.282.5396.2012. PubMed PMID: 9851916.

8. Hu PJ. Dauer. In: doi/10.1895/wormbook.1.144.1, editor. WormBook, ed The C elegans Research Community, WormBook. http://www.wormbook.org2007.

9. Bik HM, Lambshead PJD, Thomas WK, Lunt DH. Moving towards a complete molecular framework of the Nematoda: a focus on the Enoplida and early-branching clades. BMC Evol Biol. 2010;10:353-. doi: 10.1186/1471-2148-10-353. PubMed PMID: 21073704.

10. Blaxter M, Koutsovoulos G. The evolution of parasitism in Nematoda. Parasitology. 2015;142 Suppl 1(Suppl 1):S26-S39. Epub 06/25. doi: 10.1017/S0031182014000791. PubMed PMID: 24963797.

11. Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, et al. A molecular evolutionary framework for the phylum Nematoda. Nature. 1998;392(6671):71-5. Epub 1998/03/24. doi: 10.1038/32160. PubMed PMID: 9510248.

12. Weinstein SB, Kuris AM. Independent origins of parasitism in Animalia. Biology letters. 2016;12(7). Epub 2016/07/21. doi: 10.1098/rsbl.2016.0324. PubMed PMID: 27436119; PubMed Central PMCID: PMCPMC4971171.

13. Windsor DA. Most of the species on Earth are parasites. Int J Parasitol. 1998;28(12):1939-41. Epub 1999/01/30. doi: 10.1016/s0020-7519(98)00153-2. PubMed PMID: 9925276.

14. Viney M. How Can We Understand the Genomic Basis of Nematode Parasitism? Trends in parasitology. 2017;33(6):444-52. Epub 2017/03/10. doi: 10.1016/j.pt.2017.01.014. PubMed PMID: 28274802; PubMed Central PMCID: PMCPMC5449551.

15. Felix MA, Braendle C. The natural history of Caenorhabditis elegans. Current biology : CB. 2010;20(22):R965-9. Epub 2010/11/26. doi: 10.1016/j.cub.2010.09.050. PubMed PMID: 21093785.

16. Sommer RJ. Pristionchus pacificus. WormBook, ed The C elegans Research Community, Wormbook, doi/101895/wormbook11021, http://wwwwormbookorg. 2006.

17. Murrell KD, Eriksen L, Nansen P, Slotved HC, Rasmussen T. Ascaris suum: A Revision of Its Early Migratory Path and Implications for Human Ascariasis. The Journal of parasitology. 1997;83(2):255-60. doi: 10.2307/3284450.

18. Viney ME, Lok JB. *Strongyloides* spp. WormBook, ed The *C elegans* Research Community, Wormbook, doi/101895/wormbook11411, http://wwwwormbookorg. 2007.

19. Hunt VL, Tsai IJ, Coghlan A, Reid AJ, Holroyd N, Foth BJ, et al. The genomic basis of parasitism in the *Strongyloides* clade of nematodes. Nature genetics. 2016;48(3):299-307. Epub 2016/02/02. doi: 10.1038/ng.3495. PubMed PMID: 26829753.

20. Organisation WH. Neglected Tropical Diseases. https://wwwwhoint/neglected_diseases/diseases/ en/ Accessed November 2019. 2019.

21. Organisation WH. Intestinal Worms - Strategy. https://wwwwhoint/intestinal_worms/strategy/en/ Accessed November 2019. 2019.

22. Organisation WH. WHO Model Prescribing Information - Drugs Used in Parasitic Diseases - Second Edition. https://apps.who.int/medicinedocs/en/d/Jh2922e/3.2.2.html. 1995.

23. Organisation WH. African Programme for Onchocerciasis Control (APOC) - Ivermectin. https://www.who.int/apoc/cdti/ivermectin/en/. 2019.

24. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. The Journal of clinical investigation. 2008;118(4):1311-21. doi: 10.1172/JCI34261. PubMed PMID: 18382743.

25. Nana-Djeunga HC, Bourguinat C, Pion SD, Bopda J, Kengne-Ouafo JA, Njiokou F, et al. Reproductive Status of Onchocerca volvulus after Ivermectin Treatment in an Ivermectin-Naïve and a Frequently Treated Population from Cameroon. PLOS Neglected Tropical Diseases. 2014;8(4):e2824. doi: 10.1371/journal.pntd.0002824.

26. Doyle SR, Bourguinat C, Nana-Djeunga HC, Kengne-Ouafo JA, Pion SDS, Bopda J, et al. Genome-wide analysis of ivermectin response by Onchocerca volvulus reveals that genetic drift and soft selective sweeps contribute to loss of drug sensitivity. PLOS Neglected Tropical Diseases. 2017;11(7):e0005816. doi: 10.1371/journal.pntd.0005816.

27. Lustigman S, McCarter JP. Ivermectin resistance in Onchocerca volvulus: toward a genetic basis. PLoS neglected tropical diseases. 2007;1(1):e76-e. doi: 10.1371/journal.pntd.0000076. PubMed PMID: 17989789.

28. Eberhardt AG, Mayer WE, Bonfoh B, Streit A. The *Strongyloides* (Nematoda) of sheep and the predominant Strongyloides of cattle form at least two different, genetically isolated populations. Vet Parasitol. 2008;157(1-2):89-99. Epub 2008/09/02. doi: 10.1016/j.vetpar.2008.07.019. PubMed PMID: 18760537.

29. Eberhardt AG, Mayer WE, Streit A. The free-living generation of the nematode *Strongyloides papillosus* undergoes sexual reproduction. Int J Parasitol. 2007;37(8-9):989-1000. Epub 2007/02/28. doi: 10.1016/j.ijpara.2007.01.010. PubMed PMID: 17324432.

30. Jaleta TG, Zhou S, Bemm FM, Schär F, Khieu V, Muth S, et al. Different but overlapping populations of *Strongyloides stercoralis* in dogs and humans—Dogs as a possible source for zoonotic strongyloidiasis. PLOS Neglected Tropical Diseases. 2017;11(8):e0005752. doi: 10.1371/journal.pntd.0005752.

31. Fisher MC, Viney ME. Microsatellites of the parasitic nematode Strongyloides ratti. Mol Biochem Parasitol. 1996;80(2):221-4. Epub 1996/10/01. doi: 10.1016/0166-6851(96)02699-0. PubMed PMID: 8892300.

32. Tindall NR, Wilson PA. Criteria for a proof of migration routes of immature parasites inside hosts exemplified by studies of Strongyloides ratti in the rat. Parasitology. 1988;96 (Pt 3):551-63. Epub 1988/06/01. doi: 10.1017/s0031182000080185. PubMed PMID: 3405640.

33. Kimura E, Shintoku Y, Kadosaka T, Fujiwara M, Kondo S, Itoh M. A second peak of egg excretion in Strongyloides ratti-infected rats: its origin and biological meaning. Parasitology. 1999;119 (Pt 2):221-6. Epub 1999/08/31. doi: 10.1017/s0031182099004631. PubMed PMID: 10466130.

34. Nemetschke L, Eberhardt AG, Hertzberg H, Streit A. Genetics, chromatin diminution, and sex chromosome evolution in the parasitic nematode genus *Strongyloides*. Current biology : CB. 2010;20(19):1687-96. Epub 2010/09/14. doi: 10.1016/j.cub.2010.08.014. PubMed PMID: 20832309.

35. Nutman TB. Human infection with *Strongyloides stercoralis* and other related *Strongyloides* species. Parasitology. 2017;144(3):263-73. Epub 2016/05/18. doi: 10.1017/s0031182016000834. PubMed PMID: 27181117; PubMed Central PMCID: PMCPMC5563389.

36. Grove DI. Human strongyloidiasis. Advances in parasitology. 1996;38:251-309. Epub 1996/01/01. doi: 10.1016/s0065-308x(08)60036-6. PubMed PMID: 8701797.

37. Neva FA. Biology and immunology of human strongyloidiasis. The Journal of infectious diseases. 1986;153(3):397-406. Epub 1986/03/01. doi: 10.1093/infdis/153.3.397. PubMed PMID: 3081656.

38. Yamada M, Matsuda S, Nakazawa M, Arizono N. Species-specific differences in heterogonic development of serially transferred free-living generations of *Strongyloides planiceps* and *Strongyloides stercoralis*. The Journal of parasitology. 1991;77(4):592-4. Epub 1991/08/01. PubMed PMID: 1865267.

39. Grant WN, Stasiuk S, Newton-Howes J, Ralston M, Bisset SA, Heath DD, et al. Parastrongyloides trichosuri, a nematode parasite of mammals that is uniquely suited to genetic analysis. Int J Parasitol. 2006;36(4):453-66. Epub 2006/02/28. doi: 10.1016/j.ijpara.2005.11.009. PubMed PMID: 16500655.

40. Mackerras M. Strongyloides and parastrongyloides (Nematoda: Rhabdiasoidea) in Australian Marsupials. Australian Journal of Zoology. 1959;7(2):87-104. doi: https://doi.org/10.1071/ZO9590087.

41. Kulkarni A, Dyka A, Nemetschke L, Grant WN, Streit A. *Parastrongyloides trichosuri* suggests that XX/XO sex determination is ancestral in Strongyloididae (Nematoda). Parasitology. 2013;140(14):1822-30. Epub 2013/08/21. doi: 10.1017/s0031182013001315. PubMed PMID: 23953590.

42. Felix MA, De Ley P, Sommer RJ, Frisse L, Nadler SA, Thomas WK, et al. Evolution of vulva development in the Cephalobina (Nematoda). Developmental biology. 2000;221(1):68-86. Epub 2000/04/25. doi: 10.1006/dbio.2000.9665. PubMed PMID: 10772792.

43. Speare. Identification of species of Strongyloides. In Strongyloidiasis a major roundworm infection of man. 1989.

44. Weeks SC. The role of androdioecy and gynodioecy in mediating evolutionary transitions between dioecy and hermaphroditism in the animalia.

Evolution; international journal of organic evolution. 2012;66(12):3670-86. Epub 2012/12/05. doi: 10.1111/j.1558-5646.2012.01714.x. PubMed PMID: 23206127.

45. Chaudhuri J, Bose N, Tandonnet S, Adams S, Zuco G, Kache V, et al. Mating dynamics in a nematode with three sexes and its evolutionary implications. Scientific Reports. 2015;5:17676. doi: 10.1038/srep17676 https://www.nature.com/articles/srep17676 - supplementary-information.

46. Streit A. How to become a parasite without sex chromosomes: a hypothesis for the evolution of *Strongyloides* spp. and related nematodes. Parasitology. 2014;141(10):1244-54. Epub 2014/05/16. doi: 10.1017/s003118201400064x. PubMed PMID: 24829037.

47. Streit A. Genetics: modes of reproduction and genetic analysis. Parasitology. 2016:1-11. Epub 2016/03/24. doi: 10.1017/s0031182016000342. PubMed PMID: 27003130.

48. Harvey SC, Gemmill AW, Read AF, Viney ME. The control of morph development in the parasitic nematode *Strongyloides ratti*. Proceedings of the Royal Society of London Series B: Biological Sciences. 2000;267(1457):2057-63. doi: 10.1098/rspb.2000.1249.

49. Crook M, Viney ME. The effect of non-immune stresses on the development of Strongyloides ratti. Parasitology. 2005;131(Pt 3):383-92. Epub 2005/09/24. doi: 10.1017/s0031182005007675. PubMed PMID: 16178360.

50. Harvey SC, Viney ME. Sex determination in the parasitic nematode Strongyloides ratti. Genetics. 2001;158(4):1527-33. Epub 2001/08/22. PubMed PMID: 11514444; PubMed Central PMCID: PMCPMC1461766.

51. Albertson DG, Nwaorgu OC, Sulston JE. Chromatin diminution and a

chromosomal mechanism of sexual differentiation in Strongyloides papillosus. Chromosoma. 1979;75(1):75-87. Epub 1979/10/02. doi: 10.1007/bf00330626. PubMed PMID: 533664.

52. Streit A, Wang J, Kang Y, Davis RE. Gene silencing and sex determination by programmed DNA elimination in parasitic nematodes. Curr Opin Microbiol. 2016;32:120-7. Epub 06/14. doi: 10.1016/j.mib.2016.05.012. PubMed PMID: 27315434.

53. Wang J, Davis RE. Programmed DNA elimination in multicellular organisms. Curr Opin Genet Dev. 2014;27:26-34. Epub 06/02. doi: 10.1016/j.gde.2014.03.012. PubMed PMID: 24886889.

54. Viney ME, Green LD, Brooks JA, Grant WN. Chemical mutagenesis of the parasitic nematode *Strongyloides ratti* to isolate ivermectin resistant mutants. International Journal for Parasitology. 2002;32(14):1677-82. doi: https://doi.org/10.1016/S0020-7519(02)00157-1.

55. Grant WN, Skinner SJ, Newton-Howes J, Grant K, Shuttleworth G, Heath DD, et al. Heritable transgenesis of Parastrongyloides trichosuri: a nematode parasite of mammals. Int J Parasitol. 2006;36(4):475-83. Epub 2006/02/28. doi: 10.1016/j.ijpara.2005.12.002. PubMed PMID: 16500659.

56. Lok JB, Shao H, Massey HC, Li X. Transgenesis in *Strongyloides* and related parasitic nematodes: historical perspectives, current functional genomic applications and progress towards gene disruption and editing. Parasitology. 2016;144(3):327-42. Epub 03/22. doi: 10.1017/S0031182016000391.

57. Lok JB, Massey HC, Jr. Transgene expression in Strongyloides stercoralis following gonadal microinjection of DNA constructs. Mol Biochem Parasitol. 2002;119(2):279-84. Epub 2002/01/30. doi: 10.1016/s0166-6851(01)00414-5. PubMed PMID: 11814580.

58. Li X, Massey HC, Jr., Nolan TJ, Schad GA, Kraus K, Sundaram M, et al. Successful transgenesis of the parasitic nematode Strongyloides stercoralis requires endogenous non-coding control elements. Int J Parasitol. 2006;36(6):671-9. Epub 2006/02/28. doi: 10.1016/j.ijpara.2005.12.007. PubMed PMID: 16500658.

59. Li X, Shao H, Junio A, Nolan TJ, Massey HC, Jr., Pearce EJ, et al. Transgenesis in the parasitic nematode Strongyloides ratti. Molecular and biochemical parasitology. 2011;179(2):114-9. Epub 06/23. doi: 10.1016/j.molbiopara.2011.06.002. PubMed PMID: 21723330.

60. Evans TC. Transformation and Microinjection (April 6, 2006). WormBook, ed The C elegans Research Community, Wormbook, doi/101895/wormbook11081, http://wwwwormbookorg. 2006.

61. Mello CC, Fire A. Chapter 19 - DNA Transformation. Methods in Cell Biology. 1995;48:451-82.

62. Fraser MJ, Smith GE, Summers MD. Acquisition of Host Cell DNA Sequences by Baculoviruses: Relationship Between Host DNA Insertions and FP Mutants of Autographa californica and Galleria mellonella Nuclear Polyhedrosis Viruses. J Virol. 1983;47(2):287-300. PubMed PMID: 16789244.

63. Junio AB, Li X, Massey HC, Jr., Nolan TJ, Todd Lamitina S, Sundaram MV, et al. Strongyloides stercoralis: cell- and tissue-specific transgene expression and co-transformation with vector constructs incorporating a common multifunctional 3' UTR. Experimental parasitology. 2008;118(2):253-65. Epub 09/14. doi: 10.1016/j.exppara.2007.08.018. PubMed PMID: 17945217.

64. Shao H, Li X, Nolan TJ, Massey HC, Jr., Pearce EJ, Lok JB. Transposon-mediated chromosomal integration of transgenes in the parasitic nematode *Strongyloides ratti* and establishment of stable transgenic lines. PLoS Pathog.

2012;8(8):e1002871. Epub 2012/08/23. doi: 10.1371/journal.ppat.1002871. PubMed PMID: 22912584; PubMed Central PMCID: PMCPmc3415448.

65. Castelletto ML, Massey HC, Jr., Lok JB. Morphogenesis of Strongyloides stercoralis infective larvae requires the DAF-16 ortholog FKTF-1. PLoS pathogens. 2009;5(4):e1000370-e. Epub 04/10. doi: 10.1371/journal.ppat.1000370. PubMed PMID: 19360119.

66. Stoltzfus JD, Massey HC, Jr., Nolan TJ, Griffith SD, Lok JB. Strongyloides stercoralis age-1: a potential regulator of infective larval development in a parasitic nematode. PLoS One. 2012;7(6):e38587-e. Epub 06/06. doi: 10.1371/journal.pone.0038587. PubMed PMID: 22701676.

67. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, NY). 2012;337(6096):816-21. Epub 06/28. doi: 10.1126/science.1225829. PubMed PMID: 22745249.

68. Witte H, Moreno E, Rodelsperger C, Kim J, Kim JS, Streit A, et al. Gene inactivation using the CRISPR/Cas9 system in the nematode Pristionchus pacificus. Development genes and evolution. 2015;225(1):55-62. Epub 2014/12/31. doi: 10.1007/s00427-014-0486-8. PubMed PMID: 25548084.

69. Friedland AE, Tzur YB, Esvelt KM, Colaiacovo MP, Church GM, Calarco JA. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. Nature methods. 2013;10(8):741-3. Epub 2013/07/03. doi: 10.1038/nmeth.2532. PubMed PMID: 23817069; PubMed Central PMCID: PMCPMC3822328.

70. Gang SS, Castelletto ML, Bryant AS, Yang E, Mancuso N, Lopez JB, et al. Targeted mutagenesis in a human-parasitic nematode. PLOS Pathogens. 2017;13(10):e1006675. doi: 10.1371/journal.ppat.1006675.

71. Dulovic A, Streit A. RNAi-mediated knockdown of daf-12 in the model parasitic nematode Strongyloides ratti. PLOS Pathogens. 2019;15(3):e1007705. doi: 10.1371/journal.ppat.1007705.

72. Adams S, Pathak P, Shao H, Lok JB, Pires-daSilva A. Liposome-based transfection enhances RNAi and CRISPR-mediated mutagenesis in non-model nematode systems. Sci Rep. 2019;9(1):483. Epub 2019/01/27. doi: 10.1038/s41598-018-37036-1. PubMed PMID: 30679624; PubMed Central PMCID: PMCPMC6345965.

73. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391:806. doi: 10.1038/35888 https://www.nature.com/articles/35888 - supplementary-information.

74. Baulcombe D. RNA silencing in plants. Nature. 2004;431(7006):356-63. Epub 2004/09/17. doi: 10.1038/nature02874. PubMed PMID: 15372043.

75. Hall IM, Shankaranarayana GD, Noma K, Ayoub N, Cohen A, Grewal SI. Establishment and maintenance of a heterochromatin domain. Science (New York, NY). 2002;297(5590):2232-7. Epub 2002/09/07. doi: 10.1126/science.1076466. PubMed PMID: 12215653.

76. Paddison PJ, Caudy AA, Hannon GJ. Stable suppression of gene expression by RNAi in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(3):1443-8. Epub 2002/01/31. doi: 10.1073/pnas.032652399. PubMed PMID: 11818553; PubMed Central PMCID: PMCPMC122210.

77. Kavi HH, Fernandez H, Xie W, Birchler JA. Genetics and biochemistry of RNAi in Drosophila. Current topics in microbiology and immunology.

2008;320:37-75. Epub 2008/02/14. doi: 10.1007/978-3-540-75157-1_3. PubMed PMID: 18268839.

78. Tabara H, Grishok A, Mello CC. RNAi in *C. elegans*: soaking in the genome sequence. Science (New York, NY). 1998;282(5388):430-1. Epub 1998/12/05. PubMed PMID: 9841401.

79. Bakhetia M, Charlton W, Atkinson HJ, McPherson MJ. RNA Interference of Dual Oxidase in the Plant Nematode *Meloidogyne incognita*. Molecular Plant-Microbe Interactions. 2005;18(10):1099-106. doi: 10.1094/MPMI-18-1099.

80. Urwin PE, Lilley CJ, Atkinson HJ. Ingestion of Double-Stranded RNA by Preparasitic Juvenile Cyst Nematodes Leads to RNA Interference. Molecular Plant-Microbe Interactions. 2002;15(8):747-52. doi: 10.1094/MPMI.2002.15.8.747.

81. Ratnappan R, Vadnal J, Keaney M, Eleftherianos I, O'Halloran D, Hawdon JM. RNAi-mediated gene knockdown by microinjection in the model entomopathogenic nematode *Heterorhabditis bacteriophora*. Parasites & Vectors. 2016;9(1):160. doi: 10.1186/s13071-016-1442-4.

82. Morris R, Wilson L, Sturrock M, Warnock ND, Carrizo D, Cox D, et al. A neuropeptide modulates sensory perception in the entomopathogenic nematode *Steinernema carpocapsae*. PLOS Pathogens. 2017;13(3):e1006185. doi: 10.1371/journal.ppat.1006185.

83. Blanchard A, Guégnard F, Charvet CL, Crisford A, Courtot E, Sauvé C, et al. Deciphering the molecular determinants of cholinergic anthelmintic sensitivity in nematodes: When novel functional validation approaches highlight major differences between the model *Caenorhabditis elegans* and parasitic species. PLOS Pathogens. 2018;14(5):e1006996. doi: 10.1371/journal.ppat.1006996.

84. Misra S, Gupta J, Misra-Bhattacharya S. RNA interference mediated knockdown of *Brugia malayi* UDP-Galactopyranose mutase severely affects parasite viability, embryogenesis and in vivo development of infective larvae. Parasites & vectors. 2017;10(1):34-. doi: 10.1186/s13071-017-1967-1. PubMed PMID: 28103957.

85. Viney ME, Thompson FJ. Two hypotheses to explain why RNA interference does not work in animal parasitic nematodes. Int J Parasitol. 2008;38(1):43-7. Epub 2007/11/22. doi: 10.1016/j.ijpara.2007.08.006. PubMed PMID: 18028931.

86. Somarathne M, Gunawardene Y, Chandrasekharan NV, Dassanayake RS. Development of siRNA mediated RNA interference and functional analysis of novel parasitic nematode-specific protein of *Setaria digitata*. Exp Parasitol. 2018;186:42-9. Epub 2018/02/16. doi: 10.1016/j.exppara.2018.02.001. PubMed PMID: 29448039.

87. Dalzell JJ, McVeigh P, Warnock ND, Mitreva M, Bird DM, Abad P, et al. RNAi Effector Diversity in Nematodes. PLOS Neglected Tropical Diseases. 2011;5(6):e1176. doi: 10.1371/journal.pntd.0001176.

88. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409(6818):363-6. Epub 2001/02/24. doi: 10.1038/35053110. PubMed PMID: 11201747.

89. Nakanishi K. Anatomy of RISC: how do small RNAs and chaperones activate Argonaute proteins? Wiley interdisciplinary reviews RNA. 2016;7(5):637-60. Epub 2016/05/18. doi: 10.1002/wrna.1356. PubMed PMID: 27184117; PubMed Central PMCID: PMCPMC5084781.

90. Ahlquist P. RNA-dependent RNA polymerases, viruses, and RNA silencing. Science (New York, NY). 2002;296(5571):1270-3. Epub 2002/05/23. doi: 10.1126/science.1069132. PubMed PMID: 12016304.

91. Liu Q, Paroo Z. Biochemical principles of small RNA pathways. Annual review of biochemistry. 2010;79:295-319. Epub 2010/03/09. doi: 10.1146/annurev.biochem.052208.151733. PubMed PMID: 20205586.

92. Winston WM, Sutherlin M, Wright AJ, Feinberg EH, Hunter CP. Caenorhabditis elegans SID-2 is required for environmental RNA interference. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(25):10565-70. Epub 2007/06/15. doi: 10.1073/pnas.0611282104. PubMed PMID: 17563372; PubMed Central PMCID: PMCPMC1965553.

93. Tijsterman M, May RC, Simmer F, Okihara KL, Plasterk RHA. Genes Required for Systemic RNA Interference in *Caenorhabditis elegans*. Current Biology. 2004;14(2):111-6. doi: https://doi.org/10.1016/j.cub.2003.12.029.

94. Holz A, Streit A. Gain and Loss of Small RNA Classes—Characterization of Small RNAs in the Parasitic Nematode Family Strongyloididae. Genome Biology and Evolution. 2017;9(10):2826-43. doi: 10.1093/gbe/evx197.

95. Ding L, Spencer A, Morita K, Han M. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. Molecular cell. 2005;19(4):437-47. Epub 2005/08/20. doi: 10.1016/j.molcel.2005.07.013. PubMed PMID: 16109369.

96. Zhang L, Ding L, Cheung TH, Dong MQ, Chen J, Sewell AK, et al. Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Molecular cell. 2007;28(4):598-613. Epub 2007/11/29. doi: 10.1016/j.molcel.2007.09.014. PubMed PMID: 18042455; PubMed Central PMCID: PMCPMC2186060.

97. Sommer RJ. Evolution of development in nematodes related to C. elegans. WormBook : the online review of C elegans biology. 2005:1-17. Epub 2007/12/01. doi: 10.1895/wormbook.1.46.1. PubMed PMID: 18050392; PubMed Central PMCID: PMCPMC4781599.

98. Chitwood BG, Chitwood MB. Introduction to Nematology." University Park Press, Baltimore. 1950.

99. Pires-daSilva A. Pristionchus pacificus protocols. WormBook, ed The C elegans Research Community, Wormbook, doi/101895/wormbook11142, http://wwwwormbookorg. 2013.

100. Lambie EJ. Nematode Development: Variations on a vulval theme. Current Biology. 1994;4(12):1128-30. doi: https://doi.org/10.1016/S0960-9822(00)00254-2.

101. Chasnov JR, So WK, Chan CM, Chow KL. The species, sex, and stage specificity of a Caenorhabditis sex pheromone. Proceedings of the National Academy of Sciences. 2007;104(16):6730-5. doi: 10.1073/pnas.0608050104.

102. Felix MA, Sternberg PW. Symmetry breakage in the development of one-armed gonads in nematodes. Development (Cambridge, England). 1996;122(7):2129-42. Epub 1996/07/01. PubMed PMID: 8681794.

103. Sommer RJ. Evolutionary changes of developmental mechanisms in the absence of cell lineage alterations during vulva formation in the Diplogastridae (Nematoda). Development (Cambridge, England). 1997;124(1):243-51.

104. Liu KS, Sternberg PW. Sensory regulation of male mating behavior in Caenorhabditis elegans. Neuron. 1995;14(1):79-89. Epub 1995/01/01. doi: 10.1016/0896-6273(95)90242-2. PubMed PMID: 7826644.

105. Garcia LR, Mehta P, Sternberg PW. Regulation of distinct muscle behaviors controls the C. elegans male's copulatory spicules during mating. Cell. 2001;107(6):777-88. Epub 2001/12/19. doi: 10.1016/s0092-8674(01)00600-6. PubMed PMID: 11747813.

106. Klass M, Wolf N, Hirsh D. Development of the male reproductive system and sexual transformation in the nematode Caenorhabditis elegans. Developmental biology. 1976;52(1):1-18. Epub 1976/08/01. doi: 10.1016/0012-1606(76)90002-6. PubMed PMID: 986968.

107. White-Cooper H. Tissue, cell type and stage-specific ectopic gene expression and RNAi induction in the Drosophila testis. Spermatogenesis. 2012;2(1):11-22. doi: 10.4161/spmg.19088. PubMed PMID: 22553486.

108. Lints R, Hall DH. Male reproductive system, germ line. In WormAtlas doi:10.3908/wormatlas.2.16. 2009.

109. Kimble J, Hirsh D. The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. Developmental biology. 1979;70(2):396-417. Epub 1979/06/01. doi: 10.1016/0012-1606(79)90035-6. PubMed PMID: 478167.

110. Wolf N, Hirsh D, McIntosh JR. Spermatogenesis in males of the free-living nematode, Caenorhabditis elegans. Journal of Ultrastructure Research. 1978;63(2):155-69. doi: https://doi.org/10.1016/S0022-5320(78)80071-9.

111. Hirsh D, Oppenheim D, Klass M. Development of the reproductive system of Caenorhabditis elegans. Developmental biology. 1976;49(1):200-19. Epub 1976/03/01. doi: 10.1016/0012-1606(76)90267-0. PubMed PMID: 943344.

112. Ellis RE, Stanfield GM. The regulation of spermatogenesis and sperm function in nematodes. Seminars in cell & developmental biology. 2014;29:17-30. Epub 04/06. doi: 10.1016/j.semcdb.2014.04.005. PubMed PMID: 24718317.

113. L'Hernault SW. Spermatogenesis. WormBook : the online review of C elegans biology. 2006:1-14. Epub 2007/12/01. doi: 10.1895/wormbook.1.85.1. PubMed PMID: 18050478; PubMed Central PMCID: PMCPMC4781361.

114. Shakes DC, Wu JC, Sadler PL, Laprade K, Moore LL, Noritake A, et al. Spermatogenesis-specific features of the meiotic program in *Caenorhabditis elegans*. PLoS Genet. 2009;5(8):e1000611. Epub 2009/08/22. doi: 10.1371/journal.pgen.1000611. PubMed PMID: 19696886; PubMed Central PMCID: PMCPmc2720455.

115. Stanfield GM, Villeneuve AM. Regulation of sperm activation by SWM-1 is required for reproductive success of C. elegans males. Current biology : CB. 2006;16(3):252-63. Epub 2006/02/08. doi: 10.1016/j.cub.2005.12.041. PubMed PMID: 16461278.

116. Kimble J, Ward S. Germ-line development and fertilization. The nematode Caenorhabditis elegans, ed WB Wood Cold Spring Harbor Laboratory Press, pp191-213. 1988.

117. Shakes DC, Neva BJ, Huynh H, Chaudhuri J, Pires-Dasilva A. Asymmetric spermatocyte division as a mechanism for controlling sex ratios. Nat Commun. 2011;2:157. Epub 2011/01/20. doi: 10.1038/ncomms1160. PubMed PMID: 21245838.

118. King KL, Stewart M, Roberts TM, Seavy M. Structure and macromolecular assembly of two isoforms of the major sperm protein (MSP) from the amoeboid sperm of the nematode, Ascaris suum. Journal of cell science. 1992;101 (Pt 4):847-57. Epub 1992/04/01. PubMed PMID: 1527183.

119. Bullock TL, Roberts TM, Stewart M. 2.5 A resolution crystal structure of the motile major sperm protein (MSP) of Ascaris suum. Journal of molecular biology. 1996;263(2):284-96. Epub 1996/10/25. doi: 10.1006/jmbi.1996.0575. PubMed PMID: 8913307.

120. Roberts TM, Stewart M. Role of major sperm protein (MSP) in the protrusion and retraction of Ascaris sperm. International review of cell and molecular biology. 2012;297:265-93. Epub 2012/05/23. doi: 10.1016/b978-0-12-394308-8.00007-8. PubMed PMID: 22608562.

121. Roberts TM, Pavalko FM, Ward S. Membrane and cytoplasmic proteins are transported in the same organelle complex during nematode spermatogenesis. The Journal of cell biology. 1986;102(5):1787-96. Epub 1986/05/01. doi: 10.1083/jcb.102.5.1787. PubMed PMID: 3517007; PubMed Central PMCID: PMCPMC2114225.

122. Kelleher JF, Mandell MA, Moulder G, Hill KL, L'Hernault SW, Barstead R, et al. Myosin VI is required for asymmetric segregation of cellular components during C. elegans spermatogenesis. Current biology : CB. 2000;10(23):1489-96. Epub 2000/12/15. doi: 10.1016/s0960-9822(00)00828-9. PubMed PMID: 11114515.

123. Hammond MP, Robinson RD. Chromosome complement, gametogenesis, and development of Strongyloides stercoralis. The Journal of parasitology. 1994;80(5):689-95. Epub 1994/10/01. PubMed PMID: 7931903.

124. Triantaphyllou AC, Daniel JM. Cytology, Reproduction, and Sex Determination of Strongyloides ransomi and S. papillosus. The Journal of parasitology. 1977;63(6):961-73. doi: 10.2307/3279827.

125. Kulkarni A, Lightfoot JW, Streit A. Germline organization in *Strongyloides* nematodes reveals alternative differentiation and regulation mechanisms. Chromosoma. 2015. Epub 2015/12/15. doi: 10.1007/s00412-015-0562-5. PubMed PMID: 26661737.

126. Hammond MP, Robinson RD. Endoreplication in the ovary, testis, and intestine of Strongyloides stercoralis. The Journal of parasitology. 1994;80(6):905-10. Epub 1994/12/01. PubMed PMID: 7799163.

127. Kulkarni A, Holz A, Rodelsperger C, Harbecke D, Streit A. Differential chromatin amplification and chromosome complements in the germline of Strongyloididae (Nematoda). Chromosoma. 2016;125(1):125-36. Epub 2015/07/25. doi: 10.1007/s00412-015-0532-y. PubMed PMID: 26205504.

128. Cassada RC, Russell RL. The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Developmental biology. 1975;46(2):326-42. Epub 1975/10/01. doi: 10.1016/0012-1606(75)90109-8. PubMed PMID: 1183723.

129. Golden JW, Riddle DL. A pheromone influences larval development in the nematode Caenorhabditis elegans. Science (New York, NY). 1982;218(4572):578-80. Epub 1982/11/05. doi: 10.1126/science.6896933. PubMed PMID: 6896933.

130. Golden JW, Riddle DL. The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Developmental biology. 1984;102(2):368-78. Epub 1984/04/01. doi: 10.1016/0012-1606(84)90201-x. PubMed PMID: 6706004.

131. Srinivasan J, Kaplan F, Ajredini R, Zachariah C, Alborn HT, Teal PE, et al. A blend of small molecules regulates both mating and development in Caenorhabditis elegans. Nature. 2008;454(7208):1115-8. Epub 2008/07/25. doi: 10.1038/nature07168. PubMed PMID: 18650807; PubMed Central PMCID: PMCPMC2774729.

132. Bose N, Meyer JM, Yim JJ, Mayer MG, Markov GV, Ogawa A, et al. Natural variation in dauer pheromone production and sensing supports intraspecific competition in nematodes. Current biology : CB. 2014;24(13):1536-41. doi: 10.1016/j.cub.2014.05.045. PubMed PMID: 24980503.

133. Bose N, Ogawa A, von Reuss SH, Yim JJ, Ragsdale EJ, Sommer RJ, et al. Complex small-molecule architectures regulate phenotypic plasticity in a

nematode. Angewandte Chemie (International ed in English). 2012;51(50):12438-43. Epub 2012/11/20. doi: 10.1002/anie.201206797. PubMed PMID: 23161728; PubMed Central PMCID: PMCPMC3733369.

134. Vowels JJ, Thomas JH. Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. Genetics. 1992;130(1):105-23. Epub 1992/01/01. PubMed PMID: 1732156; PubMed Central PMCID: PMCPMC1204785.

135. Wadsworth WG, Riddle DL. Developmental regulation of energy metabolism in Caenorhabditis elegans. Developmental biology. 1989;132(1):167-73. Epub 1989/03/01. doi: 10.1016/0012-1606(89)90214-5. PubMed PMID: 2917691.

136. Wolkow CA, Hall DH. Introduction to the Dauer Larvae. WormAtlas doi:103908/wormatlasXXX. 2015.

137. Klass M, Hirsh D. Non-ageing developmental variant of Caenorhabditis elegans. Nature. 1976;260(5551):523-5. doi: 10.1038/260523a0.

138. Hotez P, Hawdon J, Schad GA. Hookworm larval infectivity, arrest and amphiparatenesis: the Caenorhabditis elegans Daf-c paradigm. Parasitology today (Personal ed). 1993;9(1):23-6. Epub 1993/01/01. doi: 10.1016/0169-4758(93)90159-d. PubMed PMID: 15463660.

139. Grove DI, Northern C, Warwick A, Lovegrove FT. Loss of surface coat by Strongyloides ratti infective larvae during skin penetration: evidence using larvae radiolabelled with 67gallium. The Journal of parasitology. 1984;70(5):689-93. Epub 1984/10/01. PubMed PMID: 6512634.

140. Stasiuk SJ, Scott MJ, Grant WN. Developmental plasticity and the evolution of parasitism in an unusual nematode, Parastrongyloides trichosuri. Evodevo. 2012;3(1):1-. doi: 10.1186/2041-9139-3-1. PubMed PMID: 22214222.

141. Crook M. The dauer hypothesis and the evolution of parasitism: 20years on and still going strong. International Journal for Parasitology. 2014;44(1):1-8. doi: https://doi.org/10.1016/j.ijpara.2013.08.004.

142. Castelletto ML, Gang SS, Okubo RP, Tselikova AA, Nolan TJ, Platzer EG, et al. Diverse Host-Seeking Behaviors of Skin-Penetrating Nematodes. PLOS Pathogens. 2014;10(8):e1004305. doi: 10.1371/journal.ppat.1004305.

143. Sciacca J, Forbes WM, Ashton FT, Lombardini E, Gamble HR, Schad GA. Response to carbon dioxide by the infective larvae of three species of parasitic nematodes. Parasitology international. 2002;51(1):53-62. Epub 2002/03/07. doi: 10.1016/s1383-5769(01)00105-2. PubMed PMID: 11880227.

144. Safer D, Brenes M, Dunipace S, Schad G. Urocanic acid is a major chemoattractant for the skin-penetrating parasitic nematode Strongyloides stercoralis. Proceedings of the National Academy of Sciences. 2007;104(5):1627-30. doi: 10.1073/pnas.0610193104.

145. Haas W, Haberl B, Syafruddin, Idris I, Kersten S. Infective larvae of the human hookworms Necator americanus and Ancylostoma duodenale differ in their orientation behaviour when crawling on surfaces. Parasitology research. 2005;95(1):25-9. Epub 2004/12/23. doi: 10.1007/s00436-004-1256-8. PubMed PMID: 15614586.

146. Bargmann CI, Horvitz HR. Control of larval development by chemosensory neurons in Caenorhabditis elegans. Science (New York, NY). 1991;251(4998):1243-6. Epub 1991/03/08. doi: 10.1126/science.2006412. PubMed PMID: 2006412.

147. Gilabert A, Curran DM, Harvey SC, Wasmuth JD. Expanding the view on the evolution of the nematode dauer signalling pathways: refinement through gene gain and pathway co-option. BMC Genomics. 2016;17(1):476. doi: 10.1186/s12864-016-2770-7.

148. Paradis S, Ruvkun G. Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes & development. 1998;12(16):2488-98. Epub 1998/08/26. doi: 10.1101/gad.12.16.2488. PubMed PMID: 9716402; PubMed Central PMCID: PMCPMC317081.

149. Ma G, Wang T, Korhonen PK, Stroehlein AJ, Young ND, Gasser RB. Dauer signalling pathway model for Haemonchus contortus. Parasites & Vectors. 2019;12(1):187. doi: 10.1186/s13071-019-3419-6.

150. Crook M, Thompson FJ, Grant WN, Viney ME. daf-7 and the development of Strongyloides ratti and Parastrongyloides trichosuri. Mol Biochem Parasitol. 2005;139(2):213-23. Epub 2005/01/25. doi: 10.1016/j.molbiopara.2004.11.010. PubMed PMID: 15664656.

151. Park D, Estevez A, Riddle DL. Antagonistic Smad transcription factors control the dauer/non-dauer switch in C. elegans. Development (Cambridge, England). 2010;137(3):477-85. doi: 10.1242/dev.043752.

152. Ren P, Lim CS, Johnsen R, Albert PS, Pilgrim D, Riddle DL. Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. Science (New York, NY). 1996;274(5291):1389-91. Epub 1996/11/22. doi: 10.1126/science.274.5291.1389. PubMed PMID: 8910282.

153. Poulin R, Randhawa HS. Evolution of parasitism along convergent lines: from ecology to genomics. Parasitology. 2015;142 Suppl 1:S6-s15. Epub 2013/11/16. doi: 10.1017/s0031182013001674. PubMed PMID: 24229807; PubMed Central PMCID: PMCPMC4413784.

154. Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, Li Y, et al. Identification of Ligands for DAF-12 that Govern Dauer Formation and Reproduction in *C. elegans*. Cell. 2006;124(6):1209-23. doi: https://doi.org/10.1016/j.cell.2006.01.037.

155. Hochbaum D, Zhang Y, Stuckenholz C, Labhart P, Alexiadis V, Martin R, et al. DAF-12 Regulates a Connected Network of Genes to Ensure Robust Developmental Decisions. PLOS Genetics. 2011;7(7):e1002179. doi: 10.1371/journal.pgen.1002179.

156. Shostak Y, Yamamoto KR. Overlapping but separable determinants of DNA binding and nuclear localization map to the C-terminal end of the *Caenorhabditis elegans* DAF-12 DNA binding domain. The Journal of biological chemistry. 2005;280(8):6554-60. Epub 2004/12/22. doi: 10.1074/jbc.M412928200. PubMed PMID: 15611047.

157. Snow MI, Larsen PL. Structure and expression of *daf-12*: a nuclear hormone receptor with three isoforms that are involved in development and aging in *Caenorhabditis elegans*. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression. 2000;1494(1):104-16. doi: https://doi.org/10.1016/S0167-4781(00)00224-4.

158. Wang Z, Zhou XE, Motola DL, Gao X, Suino-Powell K, Conneely A, et al. Identification of the nuclear receptor DAF-12 as a therapeutic target in parasitic nematodes. Proceedings of the National Academy of Sciences. 2009;106(23):9138-43. doi: 10.1073/pnas.0904064106.

159. Antebi A. Nuclear receptor signal transduction in C. elegans. WormBook : the online review of C elegans biology. 2015:1-49. Epub 2015/06/13. doi: 10.1895/wormbook.1.64.2. PubMed PMID: 26069085; PubMed Central PMCID: PMCPMC5402207.

160. Antebi A, Yeh WH, Tait D, Hedgecock EM, Riddle DL. *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes & development. 2000;14(12):1512-27. Epub 2000/06/20. PubMed PMID: 10859169; PubMed Central PMCID: PMCPMC316684.

161. Matyash V, Entchev EV, Mende F, Wilsch-Bräuninger M, Thiele C, Schmidt AW, et al. Sterol-Derived Hormone(s) Controls Entry into Diapause in *Caenorhabditis elegans* by Consecutive Activation of DAF-12 and DAF-16. PLOS Biology. 2004;2(10):e280. doi: 10.1371/journal.pbio.0020280.

162. Wang Z, Stoltzfus J, You Y-j, Ranjit N, Tang H, Xie Y, et al. The Nuclear Receptor DAF-12 Regulates Nutrient Metabolism and Reproductive Growth in Nematodes. PLOS Genetics. 2015;11(3):e1005027. doi: 10.1371/journal.pgen.1005027.

163. Hammell CM, Karp X, Ambros V. A feedback circuit involving *let*-7-family miRNAs and DAF-12 integrates environmental signals and developmental timing in *Caenorhabditis elegans*. Proceedings of the National Academy of Sciences. 2009;106(44):18668-73. doi: 10.1073/pnas.0908131106.

164. Ludewig AH, Kober-Eisermann C, Weitzel C, Bethke A, Neubert K, Gerisch B, et al. A novel nuclear receptor/coregulator complex controls *C. elegans* lipid metabolism, larval development, and aging. Genes & development. 2004;18(17):2120-33. Epub 2004/08/18. doi: 10.1101/gad.312604. PubMed PMID: 15314028; PubMed Central PMCID: PMCPMC515290.

165. Liu F, He C-X, Luo L-J, Zou Q-L, Zhao Y-X, Saini R, et al. Nuclear Hormone Receptor Regulation of MicroRNAs Controls Innate Immune Responses in *C. elegans*. PLOS Pathogens. 2013;9(8):e1003545. doi: 10.1371/journal.ppat.1003545.

166. Penkov S, Kaptan D, Erkut C, Sarov M, Mende F, Kurzchalia TV. Integration of carbohydrate metabolism and redox state controls dauer larva formation in *Caenorhabditis elegans*. Nature Communications. 2015;6:8060. doi: 10.1038/ncomms9060 https://www.nature.com/articles/ncomms9060 - supplementary-information.

167. Fisher AL, Lithgow GJ. The nuclear hormone receptor DAF-12 has opposing effects on *Caenorhabditis elegans* lifespan and regulates genes repressed in multiple long-lived worms. Aging Cell. 2006;5(2):127-38. doi: doi:10.1111/j.1474-9726.2006.00203.x.

168. Ogawa A, Streit A, Antebi A, Sommer RJ. A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. Current biology : CB. 2009;19(1):67-71. Epub 2008/12/27. doi: 10.1016/j.cub.2008.11.063. PubMed PMID: 19110431; PubMed Central PMCID: PMCPmc2712670.

169. Albarqi MMY, Stoltzfus JD, Pilgrim AA, Nolan TJ, Wang Z, Kliewer SA, et al. Regulation of Life Cycle Checkpoints and Developmental Activation of Infective Larvae in *Strongyloides stercoralis* by Dafachronic Acid. PLOS Pathogens. 2016;12(1):e1005358. doi: 10.1371/journal.ppat.1005358.

170. Ma G, Wang T, Korhonen PK, Young ND, Nie S, Ang C-S, et al. Dafachronic acid promotes larval development in Haemonchus contortus by modulating dauer signalling and lipid metabolism. PLOS Pathogens. 2019;15(7):e1007960. doi: 10.1371/journal.ppat.1007960.

171. Zhi X, Zhou XE, Melcher K, Motola DL, Gelmedin V, Hawdon J, et al. Structural conservation of ligand binding reveals a bile acid-like signaling pathway in nematodes. The Journal of biological chemistry. 2012;287(7):4894-903. Epub 2011/12/16. doi: 10.1074/jbc.M111.315242. PubMed PMID: 22170062; PubMed Central PMCID: PMCPMC3281614.

172. Bento G, Ogawa A, Sommer RJ. Co-option of the hormone-signalling module dafachronic acid-DAF-12 in nematode evolution. Nature. 2010;466(7305):494-7. Epub 2010/07/02. doi: 10.1038/nature09164. PubMed PMID: 20592728.

173. Viney M, Kikuchi T. *Strongyloides ratti* and *S. venezuelensis* - rodent models of Strongyloides infection. Parasitology. 2016:1-10. Epub 2016/03/05. doi: 10.1017/s0031182016000020. PubMed PMID: 26935155.

174. Stiernagle T. Maintenance of *C. elegans*. WormBook, ed The *C elegans* Research Community, Wormbook, doi/101895/wormbook11011, http://wwwwormbookorg. 2006.

175. Antebi A, Culotti JG, Hedgecock EM. *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. Development (Cambridge, England). 1998;125(7):1191-205. Epub 1998/05/09. PubMed PMID: 9477318.

176. Grosmaire M, Launay C, Siegwald M, Brugière T, Estrada-Virrueta L, Berger D, et al. Males as somatic investment in a parthenogenetic nematode. Science (New York, NY). 2019;363(6432):1210-3. doi: 10.1126/science.aau0099.

177. Richter KN, Revelo NH, Seitz KJ, Helm MS, Sarkar D, Saleeb RS, et al. Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. EMBO J. 2018;37(1):139-59. Epub 2017/11/18. doi: 10.15252/embj.201695709. PubMed PMID: 29146773; PubMed Central PMCID: PMCPMC5753035.

178. Viney ME, Matthews BE, Walliker D. On the biological and biochemical nature of cloned populations of *Strongyloides ratti*. Journal of helminthology. 1992;66(1):45-52. Epub 1992/03/01. PubMed PMID: 1469259.

179. Dulovic A, Puller V, Streit A. Optimizing culture conditions for free-living stages of the nematode parasite *Strongyloides ratti*. Experimental Parasitology. 2016;168:25-30. doi: https://doi.org/10.1016/j.exppara.2016.06.005.

180. Shaham S. Methods in Cell Biology (January 02, 2006). WormBook, ed The C elegans Research Community, Wormbook, doi/101895/wormbook1491, http://wwwwormbookorg. 2006.

181. Kosinski M, McDonald K, Schwartz J, Yamamoto I, Greenstein D. *C. elegans* sperm bud vesicles to deliver a meiotic maturation signal to distant oocytes. Development (Cambridge, England). 2005;132(15):3357-69. Epub 2005/06/25. doi: 10.1242/dev.01916. PubMed PMID: 15975936.

182. Benson G. Tandem repeats finder: a program to analyze DNA sequences. Nucleic acids research. 1999;27(2):573-80. Epub 1998/12/24. doi: 10.1093/nar/27.2.573. PubMed PMID: 9862982; PubMed Central PMCID: PMCPMC148217.

PUBLICATIONS

There are two publications that form part of this thesis. They are now printed in full. Supplementary figures can be found online.

Optimizing culture conditions for the free-living stages of the model nematode parasite *Strongyloides ratti*

Alex Dulovic, Vadim Puller and Adrian Streit

Experimental Parasitology 2016. Volume 168, September 2016, Pages 25-30.
https://doi.org/10.1016/j.exppara.2016.06.005

RNAi-mediated knockdown of *daf-12* in the model parasitic nematode *Strongyloides ratti*

Alex Dulovic and Adrian Streit

Plos Pathogens 2019.. 2019 Mar; 15(3): e1007705.
Published online 2019 Mar 29. doi: 10.1371/journal.ppat.1007705

Experimental Parasitology 168 (2016) 25–30

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research brief

Optimizing culture conditions for free-living stages of the nematode parasite *Strongyloides ratti*

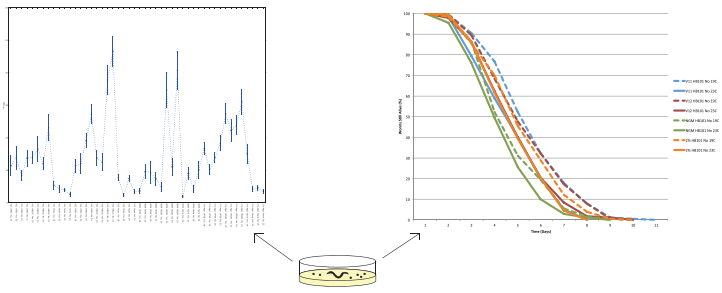
Alex Dulovic [a], Vadim Puller [b], Adrian Streit [a, *]

[a] Department of Evolutionary Biology, Max Planck Institute for Developmental Biology, Spemannstrasse 35, D-72076 Tübingen, Germany
[b] Evolutionary Dynamics and Biophysics, Max Planck Institute for Developmental Biology, Spemannstrasse 35, D-72076 Tübingen, Germany

HIGHLIGHTS

- Nematode Growth Media (NGM) plates are suboptimal for culturing *Strongyloides ratti*.
- A modified plate composition improves egg production and hatching of *S. ratti*.
- The same plates also increase longevity compared with NGM plates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 April 2016
Received in revised form
18 May 2016
Accepted 17 June 2016
Available online 19 June 2016

Keywords:
Strongyloides ratti
Culture
Nematode growth medium
Brood size
Longevity

ABSTRACT

The rat parasitic nematode *Strongyloides ratti (S. ratti)* has recently emerged as a model system for various aspects of parasite biology and evolution. In addition to parasitic parthenogenetic females, this species can also form facultative free-living generations of sexually reproducing adults. These free-living worms are bacteriovorous and grow very well when cultured in the feces of their host. However, in fecal cultures the worms are rather difficult to find for observation and experimental manipulation. Therefore, it has also been attempted to raise *S. ratti* on Nematode Growth Media (NGM) plates with *Escherichia coli* OP50 as food, exactly as described for the model nematode *Caenorhabditis elegans*.

Whilst worms did grow on these plates, their longevity and reproductive output compared to fecal cultures were dramatically reduced. In order to improve the culture success we tested other plates occasionally used for *C. elegans* and, starting from the best performing one, systematically varied the plate composition, the temperature and the food in order to further optimize the conditions. Here we present a plate culturing protocol for free-living stages of *S. ratti* with strongly improved reproductive success and longevity.

1. Introduction

Experimental manipulation of endoparasitic organisms is normally rather difficult because many if not all developmental stages, in particular the reproducing adults, reside within their respective host. However, nematode worms of the genera *Strongyloides* and *Parastrongyloides* can form facultative free-living generations in

* Corresponding author. Max-Planck-Institut für Entwicklungsbiologie, Department Evolutionary Biology, Spemannstrasse 35, D-72076 Tübingen, Germany.
E-mail addresses: alex.dulovic@tuebingen.mpg.de (A. Dulovic), vadim.puller@tuebingen.mpg.de (V. Puller), adrian.streit@tuebingen.mpg.de (A. Streit).

http://dx.doi.org/10.1016/j.exppara.2016.06.005
0014-4894/

between parasitic generations (Streit, 2008; Viney and Lok, 2015). This free-living generation offers rather unique access for the experimenter to an endo-parasite and in particular *Strongyloides ratti,* for which an excellent whole genome assembly is available (Hunt et al., 2016), has been developed as an experimental model system for basic biological and parasitological research (Viney and Kikuchi, 2016). While the parasitic adults live in the small intestines of their respective vertebrate hosts, the free-living stages are bacteriovorous. A basic set of techniques for the molecular genetic analysis of several species of *Strongyloides* and *Parastrongyloides trichosuri* (Grant et al., 2006a) has been developed over the last few years (Grant et al., 2006b; Lok, 2007; Lok and Unnasch, 2013; Nemetschke et al., 2010). Many of these techniques involve maintaining the worms on agar plates seeded with *Escherichia coli* bacteria as food. Usually, NGM (nematode growth medium) plates, known as the standard culture plates for work with the model nematode *Caenorhabditis elegans* (Stiernagle, 1999) are used. Although survival is achieved, the reproductive output of *S. ratti* free-living adults on such plates is substantially lower compared with culture in host feces (our own and other's unpublished observations). However, such worms are difficult to find for manipulation and analysis. The same likely applies for other species of *Strongyloides*, although in our hands *Strongyloides papillosus* does better on NGM plates than *S. ratti* (unpublished observation). We and others had long suspected (anecdotal evidence) that NGM plates are far from being ideal for *Strongyloides* spp. Here we systematically varied the composition of the plates, the food bacteria offered and the temperature, and measured the longevity and fecundity of free-living *S. ratti*. We demonstrate that all these traits are very strongly influenced by the exact culture conditions and we propose a recipe for agar plates on which *S. ratti* live longer and produce more eggs, a higher proportion of which develops properly, compared with NGM plates.

2. Materials and methods

2.1. S. ratti isolate and husbandry

The laboratory *S. ratti* strain ED321 was used (Viney, 1996). This strain was derived from ED5 (Viney et al., 1992) by selection for predominant development through free-living adult stages (called heterogonic or indirect) (Viney, 1996). ED321 was obtained from M. Viney, University of Bristol in 2010 and has been maintained since in our lab in female wistar rats as described (Viney et al., 1992). All experiments with animals were in accordance with international and national animal welfare guidelines and legislation. All necessary permits were granted by the local authorities (Regierungspräsidium Tübingen, AZ35/9185.82–5). The rats were kept in an in-house animal facility, which is subject to regular inspections by the local authorities (Veterinäramt Tübingen).

Four week old female wistar rats (Charles River) were infected with 500 L3i each. Seven and eight days post infection, rat feces was collected by placing the infected animals overnight in a cage, with a metal grid bottom lined with moist paper about 1 cm underneath the grid at 23 °C. Culturing in feces was done in watch glasses as described (Viney et al., 1992) at 19 °C. Under these conditions, the worms develop into infective larvae or free-living reproducing adults within three days. Worms were isolated from fecal cultures after the specified duration using the Baermann technique as follows. The fecal pellets were wrapped in Linsoft paper and placed in a funnel filled with tap water fitted with a rubber tube, closed with a clamp at the bottom at room temperature (22–23 °C). Two hours after placing the feces in the funnel, the worms that had accumulated at the bottom of the tube were collected by briefly opening the clamp. The worms were then washed in a watch glass multiple times (at least three times and until no sediment was visible) using deionized water at room temperature.

2.2. Plate preparation

Following the results of a pilot study with previously published alternative (not NGM) plates (not shown), we decided to use the recipe used by (Hart, 2006) to make plates for chemotaxis experiments in *C. elegans,* as starting point for systematic variation of the concentrations of the components. After multiple small scale trials (not shown), the best two performing plates (V11 and V12) were selected for large scale comparison with NGM. V11: 1 mM $CaCl_2$ (Sigma Aldrich), 2 mM $MgSO_4.7H_2O$ (Emsure), 5 mM KH_2PO_4 (Acros Organics), 2 mM NaCl (Sigma Aldrich), 2% w/v Agar (Serva). V12: 2 mM $CaCl_2$, 1 mM $MgSO_4.7H_2O$, 5 mM KH_2PO_4, 2 mM NaCl, 2% w/v Agar. NGM (Stiernagle, 1999): (1.7% (w/v) Agar, 50 mM NaCl, 0.25% (w/v) Peptone (BN), 1 mM $CaCl_2$, 5 μg/mL Cholesterol (Fluka), 25 mM KH_2PO_4 and 1 mM $MgSO_4$) and 2% (w/v) Agar (Serva) in water were used for comparison.

2.3. Food bacteria

E. coli HB101 in addition to *E. coli* OP50 were selected as bacteria for use in this study because they strain had previously been used to culture *S. ratti* and *Strongyloides stercoralis* (Lok and Unnasch, 2013; Shao et al., 2012). As a control, unseeded plates were also included in the study. In some experiments, the defined food was supplemented with Baermann water (as specified below), taken from the Baermann funnels the worms had been isolated with. This water contains micro organisms and other substances present in the rat feces.

2.4. Experimental treatments

Different combinations, as specified below, of plate compositions (agar), bacterial food source (bacteria), presence or absence of Baermann water (Baermann water or Bm wat.) and temperature were tested. The parameters measured were the number of eggs laid, the number of developing (hatched) progeny (for these two parameters see "2.5. Brood size determination" for details) and the longevity of the worms. Although longevity of the mothers was recorded in the "Brood size determination" experiment, longevity was measured in a second independent experiment (see 2.6. Longevity assay), which did not include a daily transfer of the worms to a new plate. All manipulations and counts involving worms on plates were done using a Zeiss Stemi 2000 stereo dissecting microscope.

2.5. Brood size determination

Plates were spotted with 100 μl of bacterial overnight culture (if required) and left to dry for four hours. Following this, gravid free-living females from 3 day old fecal cultures were isolated using the Baermann method described above. Worms were then picked singularly onto plates, each of which was subject to different conditions. If inclusion of Baermann water was part of the treatment, 150 μl of Baermann water from the fecal culture was added to the plate, immediately after the worm was picked. Plates were randomly mixed in boxes and incubated at either 19 °C or 23 °C. 24 h later, brood size was determined by manual counting with the number of eggs seen and the number of hatchlings recorded. Each box contained all agars, bacteria and Baermann water conditions. If still alive, the worm was transferred daily to a new plate with the same conditions. If Baermann water was required, it was always taken from the same stock used on the initial plate. This was

repeated every day until the worm died. Worm death was determined by lack of response to a physical stimuli (being brushed with a pick) for 2 days, upon which the initial day of no activity was recorded as the day of worm death. Alternatively, if the worm had crawled off the side of the plate (as happened on less than 8% of all plates), this was recorded as the day of death. Any alive hatched offspring were removed from the plate to avoid recounting. Once counting was complete, the plates were placed back into the incubator and then recounted every 24 h to check for newly hatched offspring. This continued until both the worm had died and no newly hatched offspring were recorded for at least 2 days. Overall, 25 plates were used for every agar, bacteria, temperature and Baermann water condition in the study.

2.6. Longevity assay

Worms were subjected to the same external environmental conditions (agars, bacteria, presence or absence of Baermann water and temperatures) as before. Plates were again spotted with 100 μl and left for 4 h to dry, after which 20 gravid 3 day female worms from a 2 h culture, were picked onto a single plate and left undisturbed for the course of the assay. Progeny were left on the plate as their difference in morphology (they all develop into L3i) did not make identification difficult. Every 24 h, the number of worms remaining alive was counted manually. If a worm was suspected of death, it was brushed lightly with a pick to check for pumping action or response. If no response or action was seen, a small X was made in the agar next to the worm. Following two further days of no activity, the worm was determined to be dead, however the day of death was recorded as being on the first day of no activity.

2.7. Statistical analysis

We analysed the data using four-way analysis of variance approach (ANOVA), where the response variable is determined by our four factors (agar, bacteria, presence or absence of Baermann water, and temperature). The response variable was either the total number of eggs, the total number of offspring or the worm lifetime. In the latter case we inferred the lifetime by fitting the time dependence of the number of worms alive by a logistic curve. This roughly corresponds to the time when the number of worms is reduced by half, and takes into account the fact that most worms survive for at least two days, after which their population sharply drops. ANOVA was used in its factor effects version i.e. the response of the dependent variable was divided into factor effects and interactions due to combinations of factors. The significance of differences between responses to different factor levels and their combinations was then tested using F-test. Pairwise comparisons of four factor combinations were done using *t*-test.

3. Results and discussion

3.1. Effect of experimental factors on egg laying

When looking solely at number of eggs laid, different agars had a significant effect upon the worm laying (p-value 4.72×10^{-9}). Of the 4 agars tested, V12 produced the highest number of eggs laid (5050, Table 1). By contrast, NGM only produced 3683 eggs. Surprisingly, a large number of eggs (4768) were still produced on 2% Agar, suggesting that *S. ratti* prefers low nutrient environments.

Bacteria when examined independently (Table 1), also had a significant effect upon laying (p-value 1.11×10^{-16}). Individuals supplied with HB101 produced significantly more eggs (9323) than those provided with either OP50 (5177) or no food (2266). The significantly lower amount of eggs laid when no food was provided is expected, as the lack of food forces the worms to reduce their reproductive output whilst they search for food. On those plates were Baermann water was added, it caused a significant (p-value 8.89×10^{-8}) reduction in laying from 9620 to 7146 eggs. Interestingly, Baermann water was seen to have the greatest positive effect when no other food had been added to the plate (Table 2). Temperature was also found to have a significant effect (p-value 1.14×10^{-6}) upon laying. The number of eggs laid was higher at 23 °C (9509) than 19 °C (7257) (Table 1).

When multiple treatments are combined, with respect to egg laying, V12 HB101 with no Baermann water at 23 °C are the optimal conditions (1166 eggs laid) (Table 2, Suppl. Fig. 1). This is 12.5% higher than the next best performing conditions. Interestingly, of the 5 best performing conditions, all 5 involve the use of HB101 as a food source, suggesting HB101 is a more suitable food source than OP50 for the maintenance of *S. ratti*. Similarly, 4 of these 5 were incubated at 23 °C, suggesting that reproductive output does increase at a higher temperature, and in the absence of Baermann water, confirming a low nutrient environment being favourable. As expected, the 5 worst performing conditions all involve no food being used, with the starvation of the worms appearing to cause a decrease in reproductive output. Surprisingly, much like the highest performing conditions, 4 of these 5 involve 23 °C being used. This higher temperature combined with the lack of food appears to severely reduce reproductive output.

3.2. Effect of experimental factors on hatched offspring production

Hatching rates are given in Table 1. The total number of hatched progeny produced, which is discussed here is the number of eggs laid which developed into infective larvae. Agar is once again having a significant effect (p-value 1.11×10^{-16}). Surprisingly the most offspring were seen on 2% agar (1224) followed by V12 agar (1213) (Table 1). It should be noted that this is because none of the single worst performing conditions involved the use of 2% agar (Table 2, Suppl. Fig. 2). By contrast only 161 offspring were seen on NGM agar, equivalent to 1/8th the number seen on 2% agar or V12, when looking at mean numbers hatched per worm. Even NGM plates which had seen high numbers of eggs laid, had significantly reduced hatching percentages when compared to similarly well performing V12 plates (i.e. 6.96% hatching on NGM HB101 without Baermann water at 23 °C and 23.50% hatching on V12 with the same other conditions (Table 2). This further supports that *S. ratti* should not be maintained on NGM.

Once again bacteria is having a significant effect (p-value 2.28×10^{-15}), with over half of all offspring been recorded on HB101 (Table 1). The particularly low numbers recorded for no food suggests that some form of enrichment is required for normal embryo development. Whilst the overall percentage of eggs that hatched is lower for HB101 than OP50 or None, this is due to the significantly higher number of eggs laid on HB101 to begin with.

The presence of Baermann water has a small effect upon hatching (p-value 0.05) with slightly more offspring seen on Baermann water free plates than on those with (Table 1).

Temperature again has a significant effect (p-value 0.01) with more offspring recorded at 23 °C than at 19 °C (Table 1), supporting the future maintenance of *S. ratti* at a higher temperature.

Again, when multiple treatments are combined, V12 agar with HB101 bacteria, no Baermann water at 23 °C are the optimal conditions, with 274 offspring recorded (Table 2). Of the 5 best performing conditions (Table 2), 3 involve the use of V12 agar suggesting it is the best performing for hatching. By contrast all 5 of the worst performing conditions use NGM agar, confirming its unsuitability as a host medium for *S. ratti*. Similarly, 4 of the 5 plates with the largest amount of hatched eggs used HB101 as a bacteria,

Table 1
Total eggs laid and hatched, and mean eggs laid and hatched per worm for each experimental variable in the Brood Size assay. For the statistical comparison of these values see Tables S1/2.

Experimental	Variable	Total eggs laid	Total eggs hatched	Hatch (%)	Mean laid eggs per worm	Mean hatched eggs per worm
Agar	V11	3245	718	22.13	10.82	2.39
	V12	5050	1213	24.02	16.83	4.04
	NGM	3683	161	4.37	12.28	0.54
	2%	4788	1224	25.56	15.96	4.08
Bacteria	Op50	5177	1075	20.76	12.94	2.69
	HB101	9323	1676	17.98	23.31	4.19
	None	2266	565	24.93	5.67	1.41
Baermann water	Present	7146	1495	20.92	11.91	2.49
	Absent	9620	1821	18.93	16.03	3.04
Temperature	19 °C	7257	1450	19.98	12.10	2.42
	23 °C	9509	1866	19.62	15.85	3.11

Table 2
Total eggs laid and mean eggs laid per worm, total eggs hatched and mean eggs hatched per worm, and the mean longevity of *S. ratti* for every experimental composition used in the Brood Size assay.

Agar	Bacteria	Temperature (°C)	Baermann water	Total eggs Laid	Total hatched eggs	Hatch (%)	Mean eggs laid per worm	Mean eggs hatched per worm	Mean lifespan
V11	Op50	19	Yes	287	59	20.56	11.48	2.36	1.84
V11	Op50	19	No	211	19	9.00	8.44	0.76	2.72
V11	Op50	23	Yes	346	176	50.87	13.84	7.04	2.12
V11	Op50	23	No	345	21	6.09	13.80	0.84	2.96
V11	HB101	19	Yes	350	46	13.14	14.00	1.84	2.52
V11	HB101	19	No	301	49	16.28	12.04	1.96	3.12
V11	HB101	23	Yes	415	202	48.67	16.60	8.08	2.04
V11	HB101	23	No	579	27	4.66	23.16	1.08	2.96
V11	None	19	Yes	136	46	33.82	5.44	1.84	1.76
V11	None	19	No	100	9	9.00	4.00	0.36	2.76
V11	None	23	Yes	109	58	53.21	4.36	2.32	1.68
V11	None	23	No	66	6	9.09	2.64	0.24	3.08
V12	Op50	19	Yes	285	53	18.71	11.40	3.60	3.32
V12	Op50	19	No	481	90	18.60	19.24	2.12	2.76
V12	Op50	23	Yes	304	77	25.33	12.16	3.08	2.72
V12	Op50	23	No	682	245	35.92	27.28	9.80	2.56
V12	HB101	19	Yes	345	37	10.72	13.80	1.48	3.28
V12	HB101	19	No	946	267	28.22	37.84	10.68	2.52
V12	HB101	23	Yes	313	16	5.11	12.52	0.64	3.12
V12	HB101	23	No	1166	274	23.50	46.64	10.96	2.64
V12	None	19	Yes	196	65	33.16	7.84	2.60	3.72
V12	None	19	No	188	25	13.30	7.52	1.00	2.56
V12	None	23	Yes	59	7	11.86	2.36	0.28	2.68
V12	None	23	No	85	57	67.06	3.40	2.28	2.92
NGM	Op50	19	Yes	92	1	1.09	3.68	0.04	2.88
NGM	Op50	19	No	237	5	2.11	9.48	0.20	2.68
NGM	Op50	23	Yes	242	14	5.79	9.68	0.56	2.76
NGM	Op50	23	No	185	2	1.08	7.40	0.08	3.64
NGM	HB101	19	Yes	125	4	3.20	5.00	0.16	2.96
NGM	HB101	19	No	278	5	1.80	11.12	0.20	2.64
NGM	HB101	23	Yes	867	17	1.96	34.68	0.68	3.28
NGM	HB101	23	No	1020	71	6.96	40.80	2.84	2.80
NGM	None	19	Yes	47	0	0.00	1.88	0.00	2.72
NGM	None	19	No	109	2	1.83	4.36	0.08	2.16
NGM	None	23	Yes	227	5	2.20	9.08	0.20	2.80
NGM	None	23	No	254	35	13.78	10.16	1.40	3.24
2%	Op50	19	Yes	418	97	23.21	16.72	3.88	2.36
2%	Op50	19	No	348	62	17.82	13.92	2.48	3.12
2%	Op50	23	Yes	256	153	59.77	10.24	1.60	2.40
2%	Op50	23	No	458	114	24.89	18.32	4.56	2.92
2%	HB101	19	Yes	686	179	26.09	27.44	7.16	2.88
2%	HB101	19	No	597	142	23.79	23.88	5.68	2.68
2%	HB101	23	Yes	557	103	18.49	22.28	4.12	2.72
2%	HB101	23	No	778	237	30.46	31.12	9.48	3.36
2%	None	19	Yes	377	153	40.58	15.08	6.12	2.56
2%	None	19	No	117	35	29.91	4.68	1.40	1.68
2%	None	23	Yes	107	40	37.38	4.28	1.60	1.72
2%	None	23	No	89	22	24.72	3.56	0.88	2.04

further demonstrating its superiority as a food source for *S. ratti*. 4 of the 5 best were kept at 23 °C whilst 4 of the 5 worst were kept at 19 °C, confirming 23 °C as the better temperature for cultivating offspring. When comparing the best treatment from our experiment (V12 HB101 No Baermann water at 23 °C) against NGM OP50 with Baermann Water at 19 °C, there was a statistically significant

Table 3
Mean and maximum Longevity of *S. ratti* for each experimental variable in the longevity assay. For the statistical comparison of these values see Tables S3.

Experimental	Variable	Mean longevity (days)	Maximum longevity (days)
Agar	V11	4.24	10
	V12	4.23	11
	NGM	3.68	8
	2%	4.00	11
Bacteria	OP50	3.98	10
	HB101	4.06	9
	None	4.08	11
Baermann water	Present	3.90	10
	Absent	4.18	11
Temperature	19 °C	4.27	11
	23 °C	3.81	10

increase in reproductive output (p-value 1.84×10^{-5}) (Supplementary Fig. S3).

One interesting observation from the study was that hatching normally increased in the absence of Baermann water when HB101 was used as the food source, but often decreased when OP50 was used (Table 2). This suggests a negative interaction may be occurring between the different bacteria found in the Baermann water and the HB101 on the plate whereas a commensal interaction may occur when OP50 is used.

Table 4
Mean and maximum longevity of *S. ratti* for every experimental composition used in the longevity assay.

Agar	Bacteria	Temperature	Baermann water	Mean longevity (days)	Maximum longevity (days)
V11	Op50	19	Yes	3.83	8
V11	Op50	19	No	4.92	9
V11	Op50	23	Yes	4.06	8
V11	Op50	23	No	3.99	7
V11	HB101	19	Yes	4.13	8
V11	HB101	19	No	4.78	9
V11	HB101	23	Yes	3.67	7
V11	HB101	23	No	4.02	6
V11	None	19	Yes	4.26	10
V11	None	19	No	4.88	10
V11	None	23	Yes	4.08	10
V11	None	23	No	4.28	8
V12	Op50	19	Yes	3.98	10
V12	Op50	19	No	4.22	10
V12	Op50	23	Yes	3.93	8
V12	Op50	23	No	4.07	7
V12	HB101	19	Yes	4.21	9
V12	HB101	19	No	4.63	8
V12	HB101	23	Yes	3.93	7
V12	HB101	23	No	4.08	8
V12	None	19	Yes	4.48	10
V12	None	19	No	5.03	11
V12	None	23	Yes	4.03	8
V12	None	23	No	4.17	7
NGM	Op50	19	Yes	3.87	7
NGM	Op50	19	No	4.11	7
NGM	Op50	23	Yes	3.36	6
NGM	Op50	23	No	3.70	7
NGM	HB101	19	Yes	4.12	8
NGM	HB101	19	No	3.95	7
NGM	HB101	23	Yes	3.12	5
NGM	HB101	23	No	3.61	7
NGM	None	19	Yes	3.61	6
NGM	None	19	No	4.18	8
NGM	None	23	Yes	3.56	7
NGM	None	23	No	2.96	7
2%	Op50	19	Yes	3.91	8
2%	Op50	19	No	4.37	9
2%	Op50	23	Yes	3.48	7
2%	Op50	23	No	3.84	7
2%	HB101	19	Yes	4.27	7
2%	HB101	19	No	4.45	8
2%	HB101	23	Yes	3.81	6
2%	HB101	23	No	4.12	6
2%	None	19	Yes	4.16	10
2%	None	19	No	4.13	11
2%	None	23	Yes	3.78	10
2%	None	23	No	3.73	8

3.3. Effect of experimental factors on longevity

As with brood size determination, agar has a significant effect (p-value 1.11×10^{-16}) upon the longevity of *S. ratti* on plates. Worms kept on V11/12 agars had the longest mean lifespan, whereas those kept on NGM had the shortest (Table 3). Similarly, the maximum lifespans seen for the worms was only 8 days on NGM, compared to 10–11 days on the V11/12 agars (Table 3). Bacteria however, unlike for brood size had little or no effect upon longevity. There were minimal differences seen in mean lifespan (Table 3) on different bacteria and those which were starved, often survived longer. It should be noted that plates often had contamination after a few days over the course of this assay. This has been seen in previous longevity assays for other species and appears to be unavoidable. In this study, nutrient rich media was more often affected by contamination than basic agar. The worms in the longevity assay lived in average about one day longer than the worms used for the brood size determination, in the process of which they were transferred to new plates every day. This indicates that repeated picking shortens lifespan, likely due to the repeated stress exerted on the worm. Unsurprisingly, Baermann water has a significant effect upon longevity (p-value 4.03×10^{-11}), with worms having a shorter mean lifespan when it was present (3.90) than when not (4.18) (Table 3). This was particularly prominent on NGM plates were the rich medium encouraged the rapid growth of any contaminating substances. This combined with the reduction in reproductive output means we recommend that Baermann water should not be added to plates in order to improve survival and reproductive output. Temperature is also having a significant effect (p-value 1.11×10^{-16}) with worms having a longer mean lifespan at 19 °C than at 23 °C (Table 3). When multiple experimental variables are examined, there are significant interactions only between Agar and Bacteria (p-value 0.03) and Baermann water and Temperature (p-value 0.01). This interaction between Baermann water and temperature is likely a negative interaction. As temperature increased, the presence of Baermann water resulted in reduced lifespans (Table 4) likely due to increased contamination. The most optimal conditions in this study were V12 with no food bacteria or Baermann water at 19 °C (5.03 days) (Table 4). The most successful conditions from the brood size assay (V12 HB101 at 23 °C without Baermann water) had mean longevity of 4.08, slightly above average for the study. By contrast, the lab standard NGM OP50 With Baermann water at 19 °C had an average of 3.87 (Table 4).

3.4. Conclusions

The results of this study show that the best conditions tested for the maintenance of *S. ratti* is V12 Agar, HB101 bacteria as a food source at 23 °C in the absence of Baermann water. V12 agar, with HB101 bacteria as a food source in the absence of Baermann water was also best performing conditions amongst all of those conducted at 19 °C. Although other experimental conditions did occasionally produce slightly better results, they were only an improvement on a single aspect for maintenance.

Acknowledgments

This work was funded by the Max Planck Society. We thank Ann-Julika Heger for her work on the study and Dorothee Harbecke for excellent technical support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exppara.2016.06.005.

References

Grant, W.N., Stasiuk, S., Newton-Howes, J., Ralston, M., Bisset, S.A., Heath, D.D., Shoemaker, C.B., 2006a. *Parastrongyloides trichosuri*, a nematode parasite of mammals that is uniquely suited to genetic analysis. Int. J. Parasitol. 36, 453–466.

Grant, W.N., Skinner, S.J., Howes, J.N., Grant, K., Shuttleworth, G., Heath, D.D., Shoemaker, C.B., 2006b. Heritable transgenesis of *Parastrongyloides trichosuri*: a nematode parasite of mammals. Int. J. Parasitol. 36, 475–483.

Hart, A., 2006. Behavior (July 3, 2006), In: Community, T.C.e.R. (Ed.) WormBook. WormBook, doi/10.1895/wormbook.1.87.1, http://www.wormbook.org.

Hunt, V.L., Tsai, I.J., Coghlan, A., Reid, A.J., Holroyd, N., Foth, B.J., Tracey, A., Cotton, J.A., Stanley, E.J., Beasley, H., Bennett, H.M., Brooks, K., Harsha, B., Kajitani, R., Kulkarni, A., Harbecke, D., Nagayasu, E., Nichol, S., Ogura, Y., Quail, M.A., Randle, N., Xia, D., Brattig, N.W., Soblik, H., Ribeiro, D.M., Sanchez-Flores, A., Hayashi, T., Itoh, T., Denver, D.R., Grant, W., Stoltzfus, J.D., Lok, J.B., Murayama, H., Wastling, J., Streit, A., Kikuchi, T., Viney, M., Berriman, M., 2016. The genomic basis of parasitism in the *Strongyloides* clade of nematodes. Nat. Genet. 48, 299–307.

Lok, J.B., 2007. Strongyloides stercoralis: a model for translational research on parasitic nematode biology (February 17, 2007), In: Community, T.C.e.R. (Ed.) WormBook WormBook, doi/10.1895/wormbook.1.134.1, http://www.wormbook.org.

Lok, J.B., Unnasch, T.R., 2013. Transgenesis in animal parasitic nematodes: Strongyloides spp. and Brugia spp. (September 30, 2013), In: Community, T.C.e.R. (Ed.) WormBook WormBook, doi/10.1895/wormbook.1.163.1, http://www.wormbook.org.

Nemetschke, L., Eberhardt, A.G., Viney, M.E., Streit, A., 2010. A genetic map of the animal-parasitic nematode *Strongyloides ratti*. Mol. Biochem. Parasitol. 169, 124–127.

Shao, H., Li, X., Nolan, T.J., Massey Jr., H.C., Pearce, E.J., Lok, J.B., 2012. Transposon-mediated chromosomal integration of transgenes in the parasitic nematode *Strongyloides ratti* and establishment of stable transgenic lines. PLOS Pathog. 8, e1002871.

Stiernagle, T., 1999. Maintenance of *C. elegans*. In: Hope, I.A. (Ed.), *C. elegans* a Practical Approach. Oxford University Press, Oxford, pp. 51–67.

Streit, A., 2008. Reproduction in *Strongyloides* (Nematoda): a life between sex and parthenogenesis. Parasitol 135, 285–294.

Viney, M., Kikuchi, T., 2016. *Strongyloides ratti* and *S. venezuelensis* - rodent models of *Strongyloides* infection. Parasitol. http://dx.doi.org/10.1017/S0031182016000020 (in press).

Viney, M.E., 1996. Developmental switching in the parasitic nematode *Strongyloides ratti*. Proc. R. Soc. - Biol. Sci. 263, 201–208.

Viney, M.E., Lok, J.B., 2015. The biology of Strongyloides spp. (July 16, 2015), In: Community, T.C.e.R. (Ed.) WormBook. WormBook, doi/10.1895/wormbook.1.141.2, http://www.wormbook.org.

Viney, M.E., Matthews, B.E., Walliker, D., 1992. On the biological and biochemical nature of cloned populations of *Strongyloides ratti*. J. Helminthol. 66, 45–52.

PLOS PATHOGENS

RESEARCH ARTICLE

RNAi-mediated knockdown of *daf-12* in the model parasitic nematode *Strongyloides ratti*

Alex Dulovic, Adrian Streit*

Department of Integrative Evolutionary Biology, Max Planck Institute for Developmental Biology, Tübingen, Baden-Württemberg, Germany

* adrian.streit@tuebingen.mpg.de

Citation: Dulovic A, Streit A (2019) RNAi-mediated knockdown of *daf-12* in the model parasitic nematode *Strongyloides ratti*. PLoS Pathog 15(3): e1007705. https://doi.org/10.1371/journal.ppat.1007705

Editor: James B. Lok, University of Pennsylvania, UNITED STATES

Received: January 7, 2019

Accepted: March 13, 2019

Published: March 29, 2019

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was funded by the Max Planck Society (AD and AS) (https://www.mpg.de/de). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

The gene *daf-12* has long shown to be involved in the dauer pathway in *Caenorhabditis elegans (C. elegans)*. Due to the similarities of the dauer larvae of *C. elegans* and infective larvae of certain parasitic nematodes such as *Strongyloides* spp., this gene has also been suspected to be involved in the development of infective larvae. Previous research has shown that the application of dafachronic acid, the steroid hormone ligand of DAF-12 in *C. elegans*, affects the development of infective larvae and metabolism in *Strongyloides*. However, a lack of tools for either forward or reverse genetics within *Strongyloides* has limited studies of gene function within these important parasites. After determining whether *Strongyloides* had the requisite proteins for RNAi, we developed and report here the first successful RNAi by soaking protocol for *Strongyloides ratti (S. ratti)* and use this protocol to study the functions of *daf-12* within *S. ratti*. Suppression of *daf-12* in *S. ratti* severely impairs the formation of infective larvae of the direct cycle and redirects development towards the non-infective (non-dauer) free-living life cycle. Further, *daf-12(RNAi) S. ratti* produce slightly but significantly fewer offspring and these offspring are developmentally delayed or incapable of completing their development to infective larvae (L3i). Whilst the successful *daf-12(RNAi)* L3i are still able to infect a new host, the resulting infection is less productive and shorter lived. Further, *daf-12* knockdown affects metabolism in *S. ratti* resulting in a shift from aerobic towards anaerobic fat metabolism. Finally, *daf-12(RNAi) S. ratti* have reduced tolerance of temperature stress.

Author summary

Strongyloides ratti is a model parasitic nematode of interest for its use in understanding basic biology and the development of novel helminth therapies. However a lack of genetic tools has stymied progress, although CRISPR/Cas9 has recently been reported. After determining whether RNAi might work in *S. ratti* by profiling the RNAi pathway proteins, we developed a successful RNAi protocol, which was used to study the gene *daf-12*. In *Caenorhabditis elegans*, *daf-12* is involved in various developmental and metabolic processes, including the formation of long-living dauer larvae which are considered to be similar to the infective larvae of *Strongyloides*. Based on the external application of

dafachronic acid (the ligand of DAF-12 in *C. elegans*) and gene expression studies, it was proposed that *daf-12* has conserved functions in *Strongyloides*. Using our RNAi method, we provide the first proof of successful gene knockdown within *S. ratti* and demonstrate that *daf-12* in *S. ratti* is involved in the same processes as *C. elegans*. This supports that *daf-12* functions are conserved in distantly related nematodes and that *daf-12* is an important target for the development of novel antihelminthics.

Introduction

In order to survive, nematodes have to be able to adapt to their surrounding conditions. In the well-studied model organism *Caenorhabditis elegans*, worms are able to switch between rapid development through a third stage larvae and the development of long lived dauer third stage larvae, which enable them to survive unsatisfactory environmental conditions [1]. Similarly, certain parasitic nematodes also may alter their life cycle development based on their surrounding conditions. The progeny of parasitic *Strongyloides* spp. are able to switch between a dauer-like pathway, which results in the formation of infective third larvae (L3i) and the infection of a new host (direct cycle), and the formation of a single free-living generation through a different, rapidly developing third stage larvae (indirect cycle) [2]. For most characterized *Strongyloides* species (such as *S. ratti*), all progeny of the free-living adults develop into L3i, however for the cat-specific species (*Strongyloides planiceps*) [3], successive free-living generations are possible. For further details on the life cycle of *Strongyloides* we refer the reader to [4]. The L3i stage is believed to be homologous to the *C. elegans* dauer [4, 5]. Compared with the *C. elegans* dauer larvae, the mechanisms that control the formation of the *Strongyloides* spp. L3i is currently rather poorly understood.

Parasitic nematodes currently constitute a great threat to humankind, from causing wide scale economic loss to having direct implications on human health. One of the more dangerous of these nematodes is the parasitic roundworm *Strongyloides stercoralis*, which infects more than 350 million people [6]. Due to the occurrence of consecutive autoinfective cycles, chronic *S. stercoralis* infections can sustain themselves for years. While individuals infected with chronic strongyloidiasis are often asymptomatic or experience mild gastro-intestinal symptoms because they control the infection at a very low level, in immuno-compromised patients the auto infective cycles may self-enhance, a condition known as hyperinfection syndrome followed by disseminated strongyloidiasis, which is normally fatal [7]. While chronic or early stages of hyperinfecting *Strongyloides* can currently be treated with ivermectin or, to some extent, other antihelminthics [8], disseminated strongyloidiasis is often fatal in spite of successful killing of the worms because the organ damage was already too great and/or the masses of dying migrating larvae cause additional damage [8–10]. In addition, resistance towards the current antihelminthics used has been identified in animals and humans, meaning new treatment options to treat strongyloidiasis are necessary [11]. Of particular interest in developing new treatments are the molecular mechanisms associated with the formation of infective larvae, because prevention of the formation of autoinfective larvae could break the continuous autoinfective cycle responsible for the severe pathology of *S. stercoralis*.

In *C. elegans*, the nuclear hormone receptor DAF-12 is known to be involved as a key regulator in the dauer pathway [12] and to act as a convergence of the pathways regulating larval diapause, developmental age and adult longevity [13, 14]. The ligand for DAF-12 in *C. elegans* is dafachronic acid (DA) [15]. In its name giving function (daf: dauer formation defective), the receptor ligand interaction appears phenotypically inhibitory because the effects of mutating

the receptor or exogenous application of DA are identical, namely inhibition of dauer formation [15]. However, DAF-12 has functions in both developmental pathways and at various time points during the ontogeny of a worm [1,13]. In addition to ligand binding, DAF-12 has been shown to interact with partners such as *daf-16* [16–18] and *din-1* [19, 20] to control developmental events and with *let-7* micro RNAs to mediate the immune response [21]. DAF-12 has also been implicated in several metabolic processes [14] such as the production of cytosolic NADPH [17] or fat metabolism [16]. Multiple genes involved in post-embryonic development have been shown to be regulated by DAF-12. In some cases, the same gene can be either repressed or activated, depending on developmental stage [15]. Further, different mutant alleles of *daf-12* within *C. elegans* have been found to effect both fertility and response to heat stress [22].

Pharmacological experiments using exogenous DA in *Strongyloides papillosus (S. papillosus)* and *S. stercoralis* suggested that a conserved endocrine module might regulate dauer/L3i formation and metabolism in *Strongyloides* spp. [5, 16, 23–26]. In particular, DA prevented the progeny of the parasitic generation from developing into L3i directly and redirected them to form free-living stages [5, 23, 26, 27]. In *S. papillosus*, DA application caused the progeny of the free-living generation to undergo an extra free-living generation [5]. DA was also shown to induce the activation of infective L3i in *S. stercoralis* [26] and to upregulate genes involved in aerobic and downregulate genes involved in anaerobic fatty acid metabolism [16]. In all these studies the pharmacological action of DA was suggested to occur through *Strongyloides*-DAF-12, which has been identified in *S. stercoralis* [27]. However, involvement of DAF-12 has never been directly demonstrated and, given that other nuclear hormone receptors exist and the similarity of the ligand binding domains in the *C. elegans* and the *Strongyloides* proteins is limited, action through DAF-12 could not be safely assumed.

Strongyloides spp. is not only of interest as a pathogen but, in part thanks to its free-living generation, it is also an attractive model system to study a number of biological questions, as has been outlined in a recent special issue of the journal "Parasitology" [28, 29]. A number of techniques for the molecular genetic analysis have been developed over the years, such as chemical mutagenesis [30, 31], genetic mapping [32] and transgenesis [33]. The full genome sequences of four species of *Strongyloides* have also been published along with transcriptomic information [34]. However for a long time, attempts to develop methods for reverse genetic knock out or knock down of gene function in *Strongyloides* spp. failed. Very recently, successful targeted mutagenesis using the CRISPR/Cas9 system has been reported for *S. stercoralis* but the same approach worked poorly by comparison in *S. ratti* [35], the species considered the prime non-human-pathogenic model species of *Strongyloides* [36]. RNA mediated interference (RNAi), an already a widely used technique in other nematodes [37, 38] would be a highly desirable technique because it would not require the establishment of mutant lines in hosts. While RNAi has been reported and successfully used for years within many plant parasitic nematodes (such as *Meloidogyne incognita* [39], *Heterodera glycine* [40] or *Globodera pallida* [40]), entomopathogenic parasitic nematodes (such as *Heterorhabditis bacteriophora* [41] or *Steinernema carpocapsae* [42]) and more recently within animal parasitic nematodes (such as *Haemonchus contortus* [43] *Ascaris suum* [44] or *Brugia malayi* [45]), it has yet to be successful within any *Strongyloides* species. The majority of the above cited papers involved the application of dsRNA which had been previously tested in *Strongyloides* spp. leading some authors to conclude that *Strongyloides* was refractive to RNAi [46]. However recent developments have involved the use of siRNAs for generating RNAi in animal parasitic nematodes [45, 47], as this bypasses the processing steps for dsRNA which several animal parasitic nematodes lack [48].

In this study, we report the development of a reliable method for RNAi in *S. ratti*, based on the protocol published earlier for *B. malayi* [45]. We decided to use the nuclear hormone

receptor DAF-12 as a test case because the relatively numerous pharmacological studies with DA mentioned above provided us with a number of testable phenotypic expectations and made this gene a highly interesting target for knock down analysis. Our results are in full agreement with the hypothesis that the pharmacological effects seen with DA involved DAF-12, and that DAF-12 is a central part of a conserved endocrine module that controls multiple processes as different as dauer/L3i formation, fat metabolism and stress tolerance.

Results

Presence and absence of homologs of known *C. elegans* RNAi pathway genes in *Strongyloides* spp

To determine whether RNAi was likely to work within *Strongyloides*, we asked if the key proteins of the RNAi machinery in *C. elegans* (as described in [48]), are present in the *Strongyloides* spp. genomes. Since in a recently published comparison of the Argonaute family genes it had been found that members of the Argonaute families believed to be required for RNAi are present in Strongyloididae [49], this gene family was not analyzed again. For each of the selected *C. elegans* genes, we determined if homologues existed in *Strongyloides* spp. following the approach described by [48], which considers sequence similarity and domain architecture and if they were likely one to one orthologs or existed in a one (in *C. elegans* to many (two or more in *Strongyloides*)), or a many to one or a many to many relationship. A gene was only considered to be an ortholog if it contained all of the functional domains present in the *C. elegans* protein. As can be seen in Table 1, *Strongyloides* spp. have genes in all functional classes according to [48]. In each of the four *Strongyloides* species *(S. ratti*, *S. papillosus*, *S. stercoralis*

Table 1. Presence of orthologs of genes known to be involved in RNAi in *C. elegans* in *Strongyloides* spp.

	Small RNA biosynthetic proteins									**dsRNA uptake and spreading and siRNA amplification effectors**										
	drh-3	*drsh-1*	*xpo-1*	*xpo-2*	*dcr-1*	*drh-1*	*pash-1*	*rde-4*	*xpo-3*	*smg-2*	*smg-6*	*ego-1*	*rrf-3*	*rrf-1*	*rsd-2*	*rsd-3*	*sid-1*	*sid-2*	*rsd-6*	*smg-5*
SRAE	X	W	W	W	W	X	W	W		W	W	Y	W	Y		W				
SPAL	X	W	W	W	W	X	W	W		W	W	Y	W	Y		W				
SSTP	X	W	W	W	W	X	W	W		W	W	W	W	W		W				
SVE	X	W	W	W	W	X	W	W		W	W	W	W	W		W				
	Nuclear RNAi effectors																			
	mut-7	*cid-1*	*ekl1*	*afl-1*	*mes-2*	*ekl-4*	*mes-6*	*rha-1*	*ekl-6*	*zfp-1*	*mut-2*	*ekl-5*	*mes-3*	*mut-16*	*rde-2*					
SRAE			W	Y	W		W	W	W	W										
SPAL			W	Y	Y	W	W	W	W	W										
SSTP			W	Y	Y	W	W	W	W	W	W									
SVE			W	W	W	W	W	W	W	W										
	RISC proteins				**RNAi inhibitors**															
	tsn-1	*ain-1*	*vig-1*	*ain-2*	*eri-1*	*xrn-2*	*adr-2*	*xrn-1*	*adr-1*	*lin-15b*	*eri-5*	*eri-7*	*eri-3*	*eri-6*						
SRAE	W	Z	W	Z	W	Z		Z				W								
SPAL	W	Z	W	Z	W	W		W			W	W								
SSTP	W	Z	W	Z	W	Z		Z				W								
SVE	W	Z	W	Z	W	Z		Z				W								

Proteins are split into 5 groups based on function as in [48]. W—likely one to one ortholog with the *C. elegans gene*. X—many to one ortholog with multiple *C. elegans* genes having a single ortholog in *Strongyloides*. Y—one to many ortholog with a single *C. elegans* gene having multiple orthologs in *Strongyloides*. Z—many to many ortholog between *C. elegans* and *Strongyloides*. Species key–SRAE (*S. ratti)*, SPAL (*S. papillosus*), SSTP (*S. stercoralis)* and SVE (*S. venezuelensis)*. The full amino acid sequences for each of the DAF-12 proteins can be found in S1 Fig.

https://doi.org/10.1371/journal.ppat.1007705.t001

and *Strongyloides venezuelensis (S. venezuelensis))*, we found orthologs for between 29 and 31 of the individual 49 *C. elegans* genes searched for. Orthologs of 28 genes are present in all four species. This is similar to the numbers described for other parasitic nematode species [48]. For the 3 proteins, *rsd-3*, *sid-1* and *sid-2* which are described in *C. elegans* as being vital for RNA uptake and spreading [50, 51], *Strongyloides* spp., like other parasitic species in which RNAi works, only have *rsd-3* present [48]. All four genes (*tsn-1*, *ain-1*, *vig-1* and *ain-2*) known to be present in the RISC machinery [48] which is required for siRNA function, are present within all of the *Strongyloides* genomes tested suggesting that the genus as whole may be susceptible to RNAi. *Strongyloides* spp. also appear to have lost most of the *mut* family of genes with *mut-2* only present in *S. stercoralis*. The loss of these genes, particularly *mut-16* may explain why the 26G RNAs have not been observed within *Strongyloides* [49, 52]. Full sequences for all of the RNAi proteins in *Strongyloides* can be found in S1 Table and S1 Fig.

Strongyloides spp. have a *daf-12* one to one ortholog

We used both the whole *C. elegans daf-12* gene, and the heavily conserved DNA-binding domain [27, 53, 54] for tblastn/blastp searching the available *Strongyloides* spp. genomes [34]. In each of the four species we identified exactly one gene containing both the DNA binding domain (DBD) and the ligand binding domain (LBD) (SRAE_0000032100 [*S. ratti*] (e-value 2.1E-99 compared to 5.4E-30 for 2nd best gene), SPAL_0001591300 [*S. papillosus*] (e-value 9.0E-97), SSTP_0001172300 [*S. stercoralis*] (e-value 4.2E-95) and SVE_0996600 [*S. venezuelensis*] (e-value 4.6E-93) (full sequences in S2 Fig). In reverse BLAST searches all these sequences identified *daf-12* as their closest *C. elegans* homolog with a large margin (i.e. 6.3E-73 versus 1.1E-29 for the second best gene for *S. ratti*). The *S. stercoralis* gene identified is the gene previously described by [27]. In the DBD, the amino acid sequences of the four *Strongyloides* spp. genes are extremely highly conserved among themselves (100% identity) and compared with *C. elegans* (96% identity). The LBDs of the *Strongyloides* genes are also very similar (>99% identity) but, the conservation compared with *C. elegans* is lower (42% identity) (Fig 1A). From this we concluded that the *Strongyloides* genes and *C. elegans daf-12* are most likely one to one orthologs and we proceeded to knock down SRAE_0000032100 in *S. ratti*. From here on, we will refer to this gene as *Sra-daf-12*.

RNAi mediated suppression of genes within *S. ratti*

In order to study the function of *daf-12* natively within *S. ratti*, *Sra-daf-12* was disrupted by RNAi soaking. To this end, first a protocol had to be devised in order to get RNAi to work within *S. ratti*. Based upon prior knowledge gained from profiling the RNAi pathway proteins, we developed a protocol for RNAi by soaking using siRNAs starting from a published protocol for *Brugia malayi [45]*, another parasitic but phylogenetically distant nematode. By systematically varying the soaking medium, duration of soaking, addition of pharyngeal pump-inducing compounds, age of larvae used in experiment and soaking temperature, we developed the two protocols ("Early-stage" and "Late-stage") described in Materials and Methods.

To determine if soaking length had a significant effect upon RNAi, larvae were soaked using the "Late-stage" protocol for upto 4 days, with the relative expression of *daf-12* determined every 24 hours. As seen in Fig 2A, 48 hours is sufficient time to achieve a statistically significant reduction of over two-thirds in expression (relative expression level 0.31 ± 0.06 ($p<0.0001$)). As a result, all experiments in this paper used a minimum soaking time of 48 hours and upto a maximum of 96 hours.

As seen in Fig 2B, expression of *daf-12* could be reduced strongly using both the "Early-stage" (0.28 ± 0.19 ($p<0.0001$)) and "Late-stage" (0.22 ± 0.18 ($p<0.0001$)) soaking protocols.

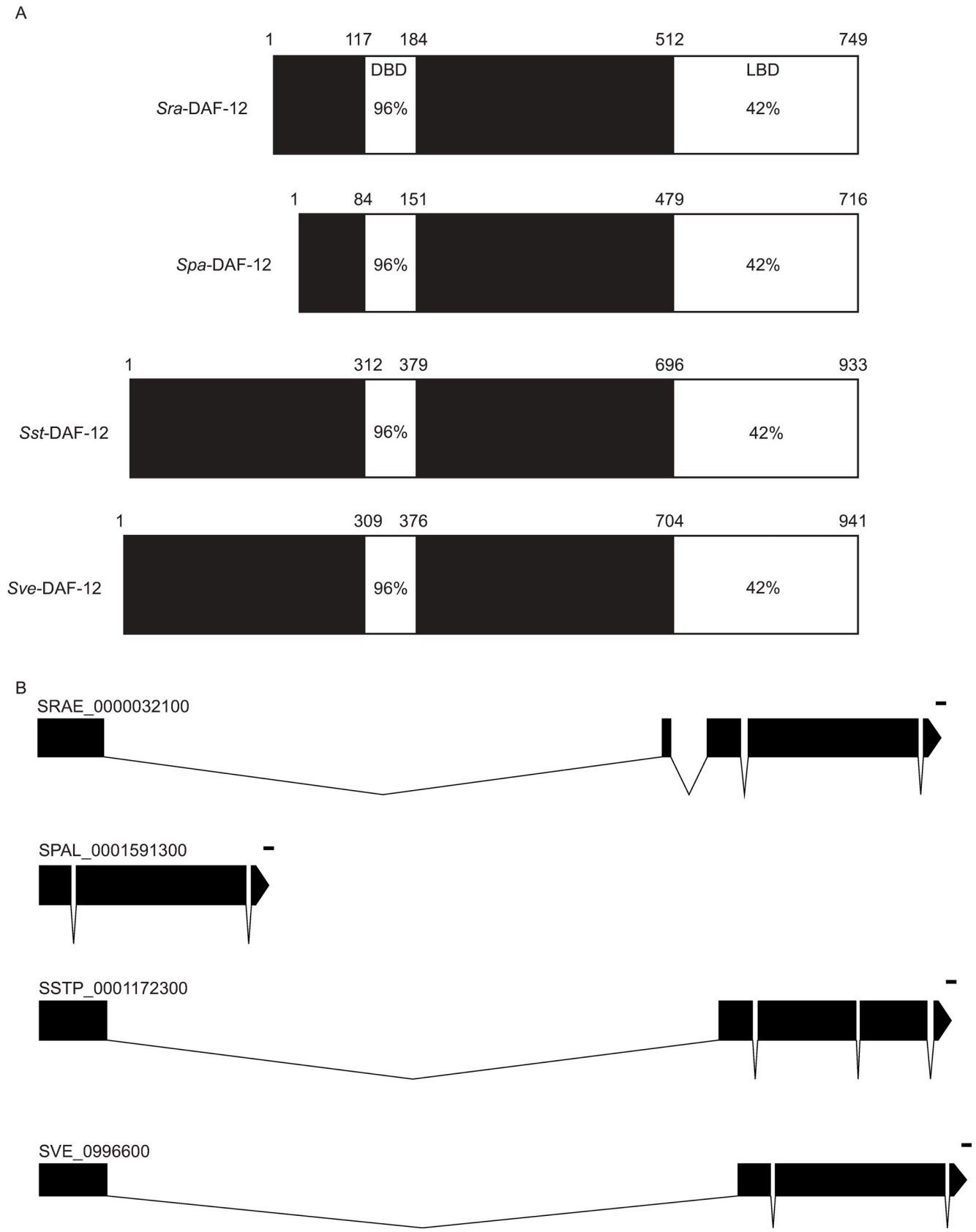


Fig 1. *daf-12* homologs in four species of *Strongyloides*. A) Schematic representation of the DAF-12 proteins. Amino acid identities with *C. elegans* are given for the DNA binding domain (DBD) and for the ligand binding domain (LBD). The species prefixes are *Sra S. ratti*, *Spa S. papillosus*, *Sst S. stercoralis*

and *Sve S. venezuelensis*. B) Gene models of the *daf-12* homologs in *Strongyloides*. spp. Notice that, *daf-12* within *S. ratti*, *S. stercoralis* and *S. venezuelensis* all have a large intron between their first and second exons unlike *S. papillosus* which lacks this feature. However the size of the *S. papillosus* protein (719 amino acids) is still similar to that of *S. ratti* (749 amino acids). The labels are the gene identifiers according to WormBase ParaSite (https://parasite.wormbase.org/index.html) containing the species codes SRAE *S. ratti*, SPAL *S. papillosus*, SSTP *S. stercoralis* and SVE *S. venezuelensis*. Scale bar = 100bp.

https://doi.org/10.1371/journal.ppat.1007705.g001

Decreased expression of *daf-12* in RNAi treated worms compared with worms treated with a scrambled siRNA (control) was determined by qRT-PCR. This suggests that the method is effective across different life stages meaning genes of interest can be studied across the entire free living part of the life cycle.

Following successful RNAi of *daf-12*, to further evaluate the method we knocked down a further two genes (*msp*[major sperm protein] and *daf-7*) using the "Late-stage" soaking protocol and compared by qRT-PCR the respective mRNA in soaked larvae treated with either a targeted siRNA or a scrambled siRNA (control). MSP is actually encoded by several very similar genes, all of which contain the targeted sequence element. For normalization we used the reference genes *tbb-1*, *rpl-37* and *gpd-2* [55]. The list of siRNA sequences used in this study can be found in Table 2 and the list of primers used can be found in S2 Table. As seen in Fig 2C, expression of the targeted gene was consistently decreased in both *daf-12* (0.22 ± 0.17 ($p<0.0001$)) and *daf-7* (0.31 ± 0.23 ($p<0.001$)). Expression could not be determined for *msp* because of the complete death of all worms and offspring treated with a *msp*-targeting siRNA. Based on *C. elegans msp* loss of function this lethality was actually the expected phenotype [56]. The fact that our method worked for three out of three cases tested confirms that the method is robust and functions across a wider-range of genes.

Finally, to determine off-target effects, all genes containing a sequence of 13 nucleotides or more identical to the siRNA had their relative expression level determined (Fig 2D). As seen in Fig 2D, minimal inconsistent changes in the mRNA levels of these genes were seen with either of the siRNAs. This suggests that the knock down was specific and that the phenotypic changes seen in the treated worms are unlikely to be the result of off-target effects but rather are the consequence of the knockdown of the gene of interest.

This flexibility in soaking stage, lack of off-target effects and the ability of study a wide variety of genes, makes RNAi potentially a highly suitable and effective tool for research in *S. ratti*.

Suppression of *daf-12* prevents direct development to L3i

As outlined in the introduction, when female larvae exit the host in *Strongyloides*, they have a choice between either undergoing direct development and developing into L3i, or alternatively undergoing indirect development with the aforementioned sexual reproduction outside of the host. The exact proportion of larvae which undergo direct development depends on both the strain and species of *Strongyloides* as well as environmental factors such as the host immune status and external conditions. For two species of *Strongyloides* (*S. stercoralis* [27] and *S. papillosus* [5]) treatment with DA prevented the formation of L3i and redirected the development towards the indirect cycle. If DA indeed acted by inactivating the L3i promoting action of *Sra*-DAF-12, knocking down *Sra-daf-12* should result in the suppression of L3i formation. To test this young L1 larvae were treated with siRNA ("Early-stage", full details in methods) and the number which then underwent direct development were counted and compared to those which underwent normal development. *daf-12(RNAi)* larvae were highly statistically significantly impaired in their ability to form direct L3i (Fig 3) with only 1.67% ± 0.94 undergoing direct development compared to 12.67% ± 2.19 in the control ($p<0.0001$).

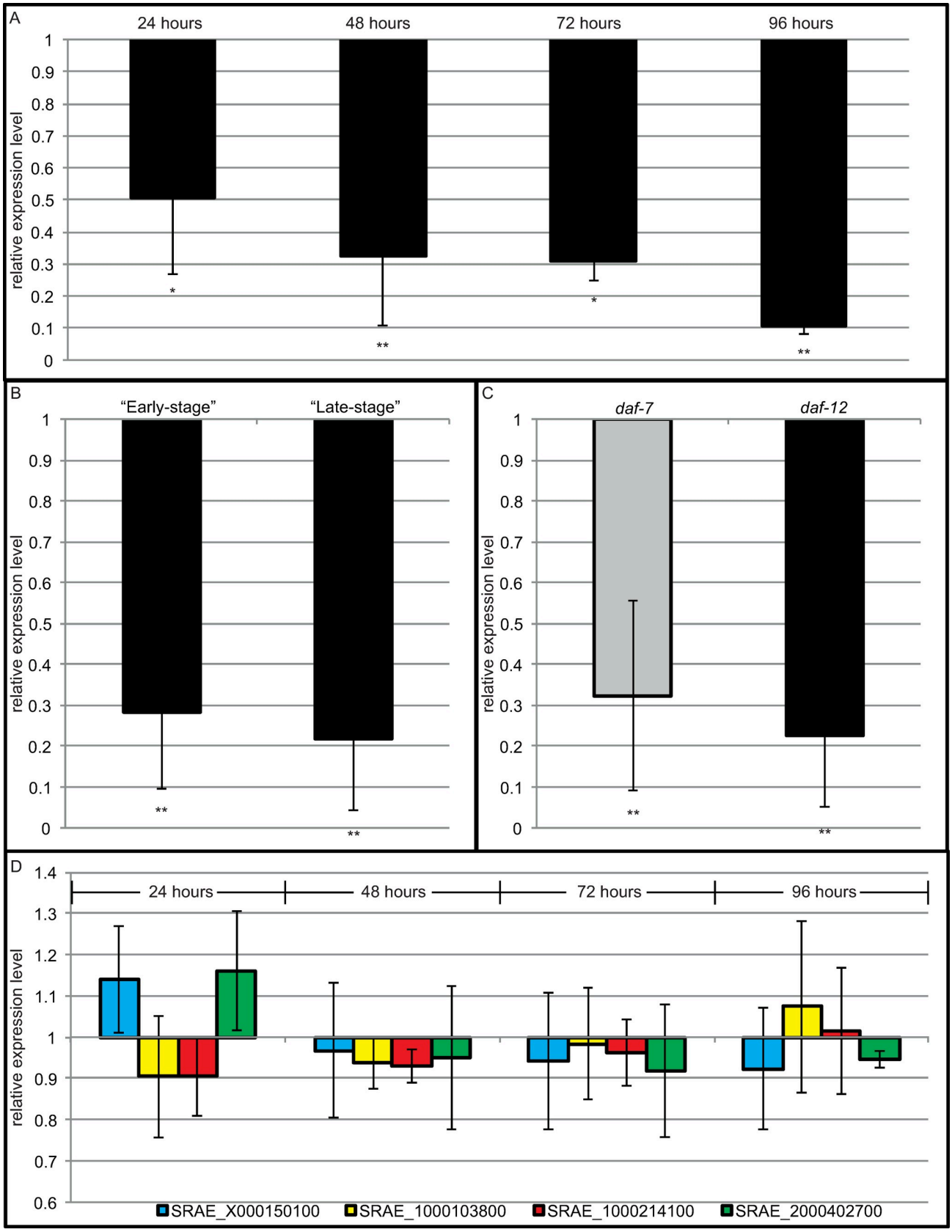

A
24 hours
48 hours
72 hours
96 hours
relative expression level
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
*
**
B
"Early-stage"
"Late-stage"
relative expression level
**
**
C
daf-7
daf-12
relative expression level
**
**
D
24 hours
48 hours
72 hours
96 hours
relative expression level
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
SRAE_X000150100
SRAE_1000103800
SRAE_1000214100
SRAE_2000402700

Fig 2. RNAi-mediated suppression within *Strongyloides ratti*. RNAi reduces expression of *daf-12* with statistically significantly increased reduction in *daf*-12 as length of soaking increases (relative gene expression of 0.51 ± 0.24 after 24 hours (p = 0.01), 0.32 ± 0.22 after 48 hours (p<0.001), 0.31 ± 0.06 after 72 hours (p = 0.01) and 0.11 ± 0.03 after 96 hours (p<0.001) (A), with both the "Early-stage" (relative gene expression 0.28 ± 0.19 (p<0.001)) and "Late-stage" soaking protocols (relative gene expression 0.22 ± 0.18 (p<0.001)) (B) resulting in a reduction following 48 hours of soaking. The "Late-stage" technique can be to achieve a statistically significant reduction in expression in both *daf-7* (in grey) (relative gene expression 0.32 ± 0.23 (p<0.001)) and *daf-12* (in black) (0.23 ± 0.17 (p<0.001)) (C), with minimal off-target effects detected across five genes with a similarity to an siRNA targeting *daf-12* in *S. ratti* over different time points (relative expression of SRAE_X000150100 was 1.14 ± 0.13 after 24 hours, 0.97 ± 0.16 after 48 hours, 0.94 ± 0.17 after 72 hours and 0.92 ± 0.15 after 96 hours. Relative expression of SRAE_1000103800 was 0.90 ± 0.15 after 24 hours, 0.94 ± 0.06 after 48 hours, 0.98 ± 0.14 after 72 hours and 1.07 ± 0.21 after 96 hours. Relative expression of SRAE_1000214100 was 0.94 ± 0.17 after 24 hours, 0.98 ± 0.14 after 48 hours, 0.96 ± 0.08 after 72 hours and 0.92 ± 0.16 after 96 hours. Relative expression of SRAE_2000402700 was 0.92 ± 0.15 after 24 hours, 1.07 ± 0.21 after 48 hours, 1.02 ± 0.15 after 72 hours and 0.95 ± 0.02 after 96 hours (no statistically significant reduction in any of genes at any of the time points)) (D). *S. ratti* larvae were soaked using either the "Early-stage" protocol (B) or "Late-stage" protocol (A-D) and then the relative expression level of the targeted gene was determined using qRT-PCR compared to 3 control genes with a scrambled siRNA used as a control. Error bars represent standard deviation. These figures show the mean value comprised of a minimum of 3 biological replicates with 3 technical replicates per biological replicate. Mann Whitney U analysis was performed to determine statistical significance. * indicates a statistically significant difference (p-value ≤0.01 - ≥0.001), ** indicates a highly statistically significant difference (p-value ≤0.001).

https://doi.org/10.1371/journal.ppat.1007705.g002

Suppression of *daf-12* has a minor effect on fecundity in *S. ratti*

To determine whether *Sra-daf-12* would affect fecundity, as is the case in *C. elegans* [19, 22], we knocked down the gene following the "Late-stage" procedure (see above and Materials and Methods).

Adults which had been soaked in *daf-12* targeting siRNAs (here on referred to as "*daf-12 (RNAi)*") had a small but statistically significant reduction in number of eggs laid (18.04 ± 1.40 per worm in control to 16.45 ± 0.96 in *daf-12(RNAi)*, p = 0.004) as seen in Fig 4. This suggests that *daf-12* in *S. ratti* may be involved in either reproductive development or alternatively in the actual laying procedure. Examination of the vulva of 10 random individuals from both treatments using high magnification (100X) DIC microscopy revealed no structural defects in the egg laying machinery. Further, we found no difference in the number of eggs still present in the worm (7.90 ± 1.10 in *daf-12(RNAi)* versus 7.50 ± 1.27 in control, p = 0.46), suggesting that the observed difference was due to a slightly smaller number of eggs produced rather than problems with egg laying.

daf-12(RNAi) progeny of free-living worms are still able to develop into L3i albeit more slowly and in reduced numbers

As DA interfered with the development of the progeny of free-living adults to L3i in two other species of *Strongyloides* [5, 26, 27], we asked if this was also the case in *daf-12(RNAi)*. The development of progeny of "Late-stage" adults (the larvae remained in RNA solution throughout development) was recorded every 24 hours over a period of 5 days post-treatment. As can be seen in Table 3, *daf-12(RNAi)* larvae were still able to develop into L3i but did so more

Table 2. List of siRNA sequences used in this study.

Targeted Gene		Sequence (5' to 3')
daf-12	sense	(UU) AGTTGATGGTCATTCACAA
	antisense	(UU) UUGUGAAUGACCAUCAACU
msp	sense	(UU) GACCUUCAGACGUGAAUGG
	antisense	(UU) CCAUUCACGUCUGAAGGUC
daf-7	sense	(UU) UGAAAUGGUACAGACAAAU
	antisense	(UU) AUUUGUCUGUACCAUUUCA
Scrambled siRNA (negative control)	sense	(UU) AGGUAGUGUAAUCGCCUUG
	antisense	(UU) CAAGGCGAUUACACUACCU

https://doi.org/10.1371/journal.ppat.1007705.t002

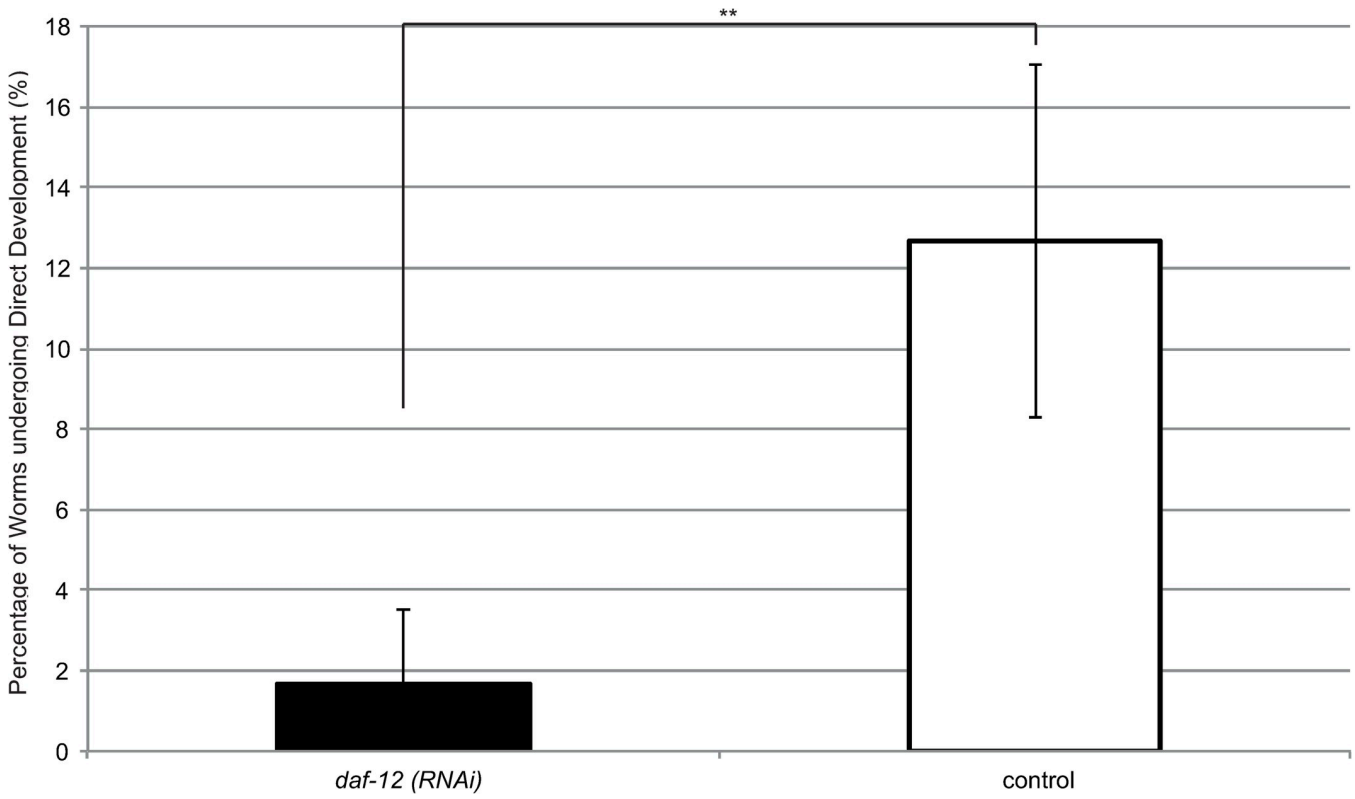


Fig 3. Suppression of *daf-12* significantly reduces the ability of worms to undergo direct development to infective larvae. Young worms were soaked with an siRNA targeting *daf-12 (daf-12(RNAi))* or a scrambled siRNA (control), and the proportion of females which developed to infective larvae after 3 days was determined. Males were excluded from this analysis as they cannot undergo direct development. Error bars represent standard deviation. This figure shows the mean value comprised from 3 biological replicates with 4 technical replicates per biological replicate. Mann Whitney U analysis was performed to determine statistical analysis. ** indicates a highly statistically significant p-value of less than 0.001 ($p<0.0001$).

https://doi.org/10.1371/journal.ppat.1007705.g003

slowly with an apparent 24 hour delay in development compared to control worms. It took 3 days post-laying until the majority of the *daf-12(RNAi)* larvae that did develop all the way through to L3i had reached this developmental stage while more than 65% of the control larvae that developed to L3i had done so after two days. Further, statistically significantly less *daf-12 (RNAi)* larvae compared to control larvae successfully completed the developed to L3i (75.36% ± 13.09 versus 91.55% ± 5.59, $p<0.0001$). Taken together these results suggest that *daf-12* does have a role in the development of the progeny of the free-living generation to infective larvae within *S. ratti*.

daf-12(RNAi) infective larvae are still able to infect rats

In order to test if the *daf-12(RNAi)* larvae that were successful in developing into morphologically normal looking L3i were functional and able to establish an infection in a new host, 100 *daf-12(RNAi)* L3i were injected subcutaneously into a rat and the infection allowed to develop for 7 days, after which the feces was collected daily and incubated at 19˚C for 7 days to allow for new L3i to develop. These L3i were counted and used as a measure for the productivity of the infection. As a control, 100 L3i which had been treated using a scrambled siRNA were also injected subcutaneously into a separate rat and the same procedures followed (full details in methods, two independent replicates were done). As can be seen in Fig 5, in two independent experiments *daf-12(RNAi)* L3i (shown in black) were still able to develop an infection within a

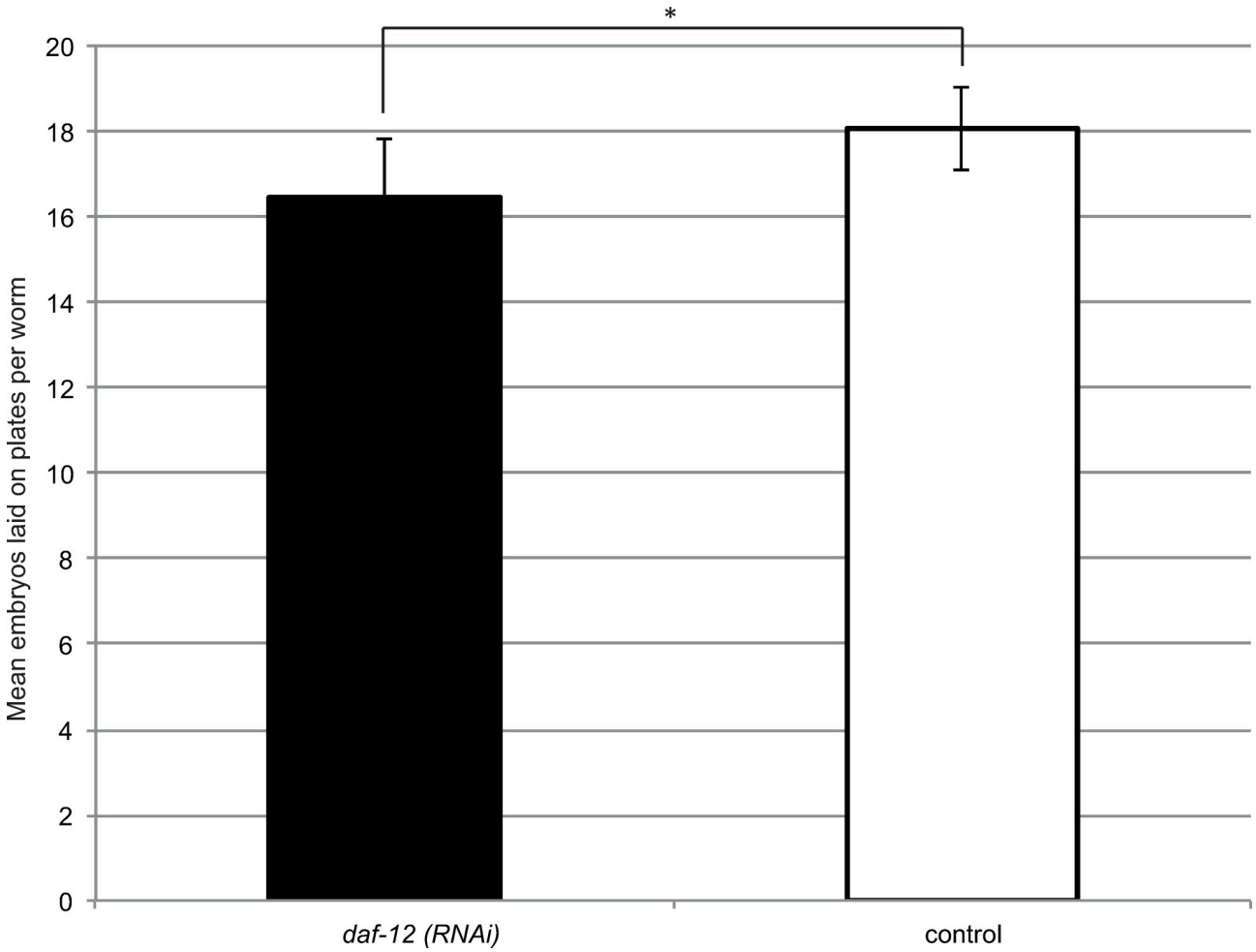


Fig 4. *daf-12(RNAi)* free-living adults lay slightly but significantly less embryos than control adults. Following soaking ("Late-stage" protocol) with either a scrambled siRNA (control) or *daf-12* targeting siRNA *(daf-12(RNAi))*, free living adults were recovered on plates and the number of embryos they laid counted. Error bars represent standard deviation. This experiment was repeated three times. The bars represent a mean calculated from all three biological replicates. Student's t-test was performed to determine statistical significance. * indicates a statistically significant p-value between 0.01 and 0.001 (p = 0.004).

https://doi.org/10.1371/journal.ppat.1007705.g004

rat, however one with a statistically significantly reduced productivity compared to the control L3i ($p<0.0001$). Knockdown of *daf-12* caused a reduced peak in worm production (average of 275 larvae after 7 days compared to 900 in the control) and the length of infection was also

Table 3. *daf-12(RNAi)* L3i develop more slowly and in fewer numbers than control L3i.

	Day 1	Day 2	Day 3	Day 4	Day 5
***daf-12 (RNAi)* L3i on each day (%)**	7.41 ± 3.97	6.17 ± 3.48	39.37 ± 11.84	19.91 ± 5.76	2.49 ± 2.00
***daf-12 (RNAi)* L3i cumulative (%)**	7.41 ± 3.97 (ns)	13.58 ± 6.28 (**)	52.95 ± 5.15 (**)	72.87 ± 12.78 (**)	75.36 ± 13.09 (**)
Control L3i on each day (%)	10.13 ± 1.77	54.75 ± 5.15	17.77 ± 4.72	7.69 ± 3.46	1.21 ± 1.50
Control L3i cumulative (%)	10.13 ± 1.77	64.88 ± 4.90	82.65 ± 7.73	90.33 ± 5.80	91.55 ± 5.59

% of larvae, which had developed to L3i at the day indicated. The numbers shown are the means of three independent replicates plus/minus the standard deviation. Per replicate at least 180 individual larvae were followed. Statistical analysis (Mann-Whitney U) to compare *daf-12 (RNAi)* and control larvae cumulatively every 24 hours was performed. ns indicates a not statistically significant value (p-value >0.01), * indicates a statistically significantly value (p-value between 0.01 and 0.001), ** indicates a highly statistically significant value (p-value <0.001)

https://doi.org/10.1371/journal.ppat.1007705.t003

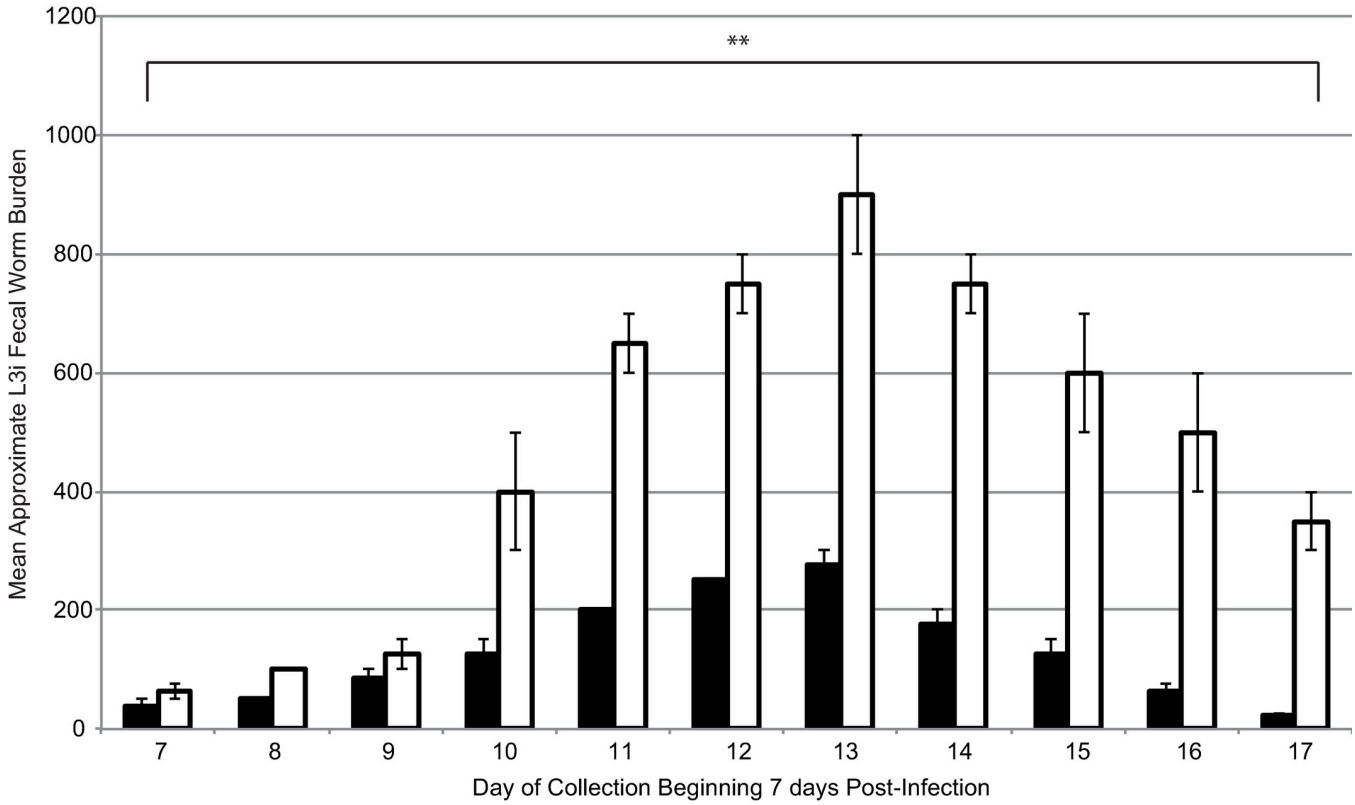


Fig 5. The *daf-12(RNAi)* larvae that do develop into L3i (black) are able to infect rats when injected subcutaneously but cause significantly less productive infections than control (scrambled siRNA) L3i (white). Approximate worm burden in the feces (as a proxi for the capacity of causing a new infection) was determined by counting the L3i present in fecal cultures after 7 days incubation at 19˚C. Fecal cultures were collected daily starting from 7 days post-infection. Error bars represent standard deviation. This experiment was carried out twice. Student's t-test was performed to determine statistical significance. ** indicates a highly statistically significant difference (p-value <0.001) between the *daf-12(RNAi)* and control infections (p<0.0001).

https://doi.org/10.1371/journal.ppat.1007705.g005

slightly reduced, with no worms being seen in fecal cultures after 17 days post-infection compared to control larvae infection in which worms could still be seen on 19 days post-infection.

Suppression of *daf-12* affects metabolism within *S. ratti*

In *C. elegans*, DA application leads to increased aerobic fat metabolism [16]. In the same publication the authors also demonstrated that exogenous DA lead to an increase in expression of genes involved in aerobic triglyceride metabolism and a lower expression of genes involved in anaerobic metabolism in *S. stercoralis*. To determine whether *Sra-daf-12* is involved in fat utilization, levels of triglycerides stored within the worms were determined and compared to levels of free glycerol (Fig 6A). Whilst free glycerol levels were similar between both *daf-12(RNAi)* and control larvae, there was a statistically significant reduction in triglycerides stored in *daf-12(RNAi)* larvae (0.011 ± 0.001 compared to 0.022 ± 0.002 in control, p<0.0001). To further examine this potential shift between aerobic and anaerobic metabolism, two genes known to be involved in aerobic metabolism (*acs-3* and *acbp-3*) and two genes known to be involved in anaerobic metabolism (*ech-8* and *acox-3*) had their relative expression levels determined by qRT-PCR (Fig 6B and 6C). *acs-3* was strongly statistically significantly downregulated (to 0.047 ± 0.029 compared to the control, p<0.001). For *acbp-3* we measured a reduction to 0.523 ± 0.259, but this was not statistically significant (p = 0.057). Similarly, both anaerobic metabolism genes were highly significantly upregulated in *daf-12(RNAi)* larvae (Fig 6C) compared to the control (5.719 ± 3.006 to 1 enrichment

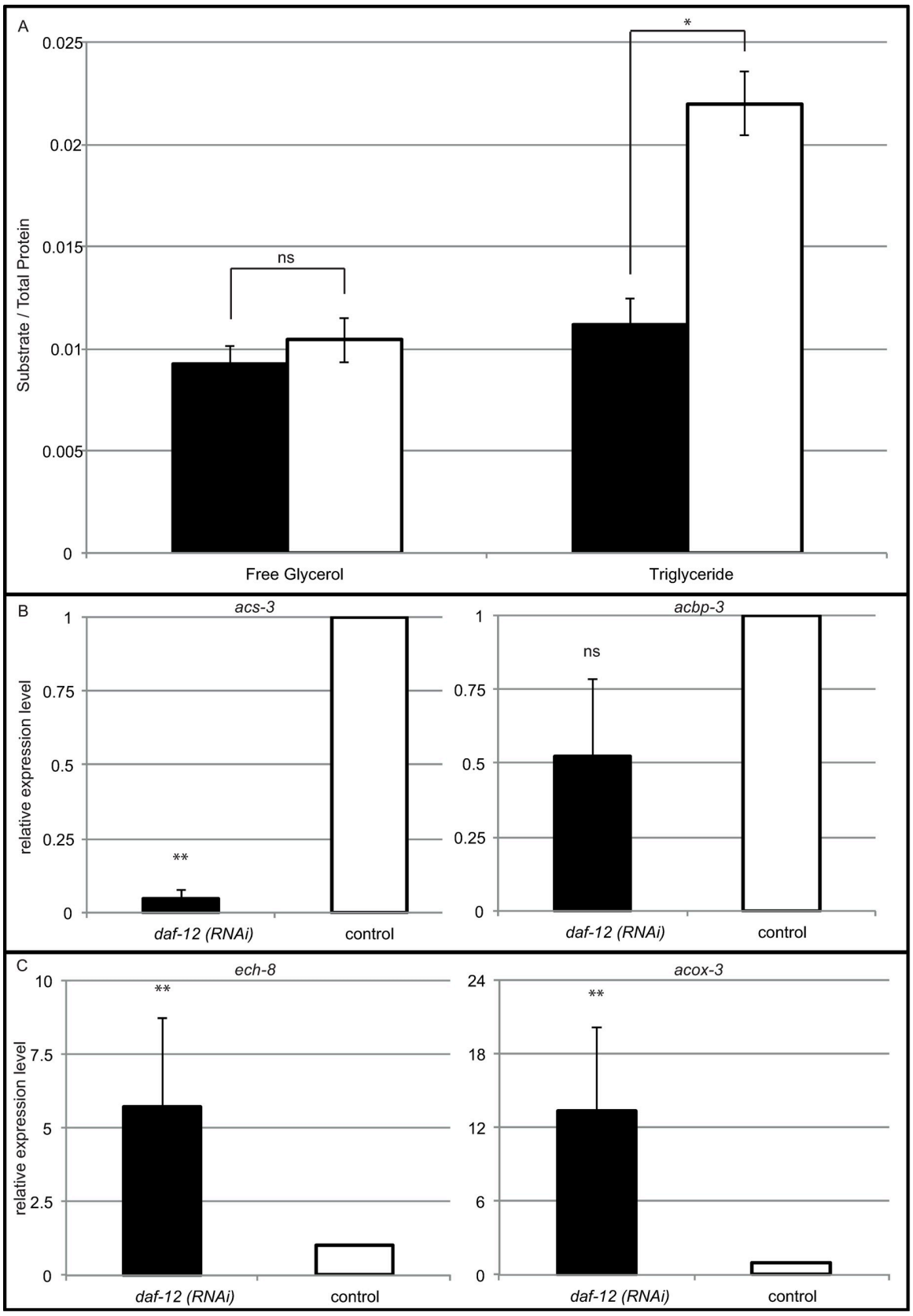

A
Substrate / Total Protein
0.025
0.02
0.015
0.01
0.005
0
ns
*
Free Glycerol
Triglyceride
B
relative expression level
acs-3
acbp-3
1
0.75
0.5
0.25
0
**
ns
daf-12 (RNAi)
control
C
relative expression level
ech-8
acox-3
10
7.5
5
2.5
0
24
18
12
6
**

Fig 6. Suppression of *daf-12* within *Strongyloides ratti* results in multiple changes in metabolism. Triglyceride content is statistically significantly reduced in *daf-12(RNAi)* larvae (black) than control larvae (white) following "Late-stage" soaking (A) (p-value <0.001). Similarly, the aerobic metabolism genes *acs-3* (p<0.001) and *acbp-3* (p = 0.057) have decreased expression in *daf-12 (RNAi)* larvae (B), while the anaerobic metabolism genes *ech-8* (p = 0.001) and *acox-3* (p<0.0001) are upregulated in *daf-12 (RNAi)* (C). To determine fatty acid stores, total triglyceride and free glycerol were measured from worm lysates and are plotted against total protein in the sample. This experiment was performed twice. To determine changes in gene expression, control larvae expression was normalized as 1 and fold change was calculated using the ΔΔCt method (see Materials and Methods) to determine change in expression of the genes of interest. Fold change shown is the mean fold change of the gene of interest compared to 3 control genes *(tbb-1*, *gpd-2*, *rpl-37* [55]) taken from at least 3 biological replicates with each biological replicate consisting of 3 technical replicates. Error bars represent standard deviation. Mann Whitney U analysis was performed to determine statistical significance. ns indicates a not statistically significant difference (p-value >0.01), * indicates a statistically significant difference (p-value ≤0.01 - ≥0.001), ** indicates a highly statistically significant difference (p-value ≤0.001).

https://doi.org/10.1371/journal.ppat.1007705.g006

in *ech-8* (p = 0.001) and 13.349 ± 6.776 to 1 in *acox-3* (p<0.001)). These results, together with [16] strongly suggest that in *Strongyloides* spp., as in *C. elegans*, ligand bound DAF-12 promotes aerobic and suppresses anaerobic metabolism.

daf-12(RNAi) are less tolerant to heat stress

Different *daf-12* mutations are known to result in different responses to heat stress in *C. elegans* [22]. To test whether *daf-12* also played a role in thermotolerance in *S. ratti*, *daf-12 (RNAi)* and control worms were subjected to different temperatures between 12˚C and 37˚C 48 hours after initiating soaking at 19˚C ("Early-stage" Protocol). The percentage that perished was used as an indicator of their tolerance to these temperatures (Fig 7). *daf-12(RNAi)* worms were significantly less tolerant to all temperatures outside of their optimum (23˚C) compared to control. Also at colder temperatures there was a statistically significantly increase in nematode death between *daf-12(RNAi)* and control (21.83% ± 6.00 in *daf-12(RNAi)* at 12˚C compared to 14.50% ± 4.91 (p = 0.003)). Interestingly at 19˚C, the survival was lower than at 23˚C despite the fact that *S. ratti* isolate used had normally been maintained at 19˚C and that the soaking experiment had been initiated at 19˚C. At 28˚C *daf-12(RNAi)* worms had a statistically highly significantly reduced survival rate (62.33% ± 7.38 dead in *daf-12(RNAi)* versus 21.92% ± 4.40 in control (p<0.001)). At 37˚C, the heat treatment was nearly completely lethal for both nematode populations, yet still a few control worms (3.33%) but no *daf-12(RNAi)* worms survived. These results suggest that *daf-12* is involved in thermotolerance within *S. ratti* and might be involved in reacting to changes in their environment.

Discussion

RNAi in *S. ratti*

We found *Strongyloides* spp. to have homologs of many but not all *C. elegans* genes known to be involved in RNAi as listed by [48]. The fact that we found essentially the same reduced set of genes in all four species of *Strongyloides* examined suggests that the reduction in gene number is real and not the consequence of incompleteness of the *Strongyloides* spp. genome assemblies. The set of putative RNAi genes present in *Strongyloides* spp. is similar to those reported in [48] for other parasitic nematodes in which RNAi works. The presence of all of the RISC protein genes (*tsn-1*, *ain-1*, *vig-1 and ain-2*) and the absence of genes such as *rsd-6*, *sid-1* or *sid-2*, all of which are known to be involved in dsRNA uptake, may explain why, for successful RNAi, we had to apply siRNAs rather than long double stranded RNAs as it is common in *C. elegans*.

Of interest is the lack of *eri*-family genes within *Strongyloides* spp. with only *eri-1* and *eri-7* present in all four genomes. These genes are known to be enhancers of RNAi [57]. The loss of *mut* genes, in particular *mut-16* in *Strongyloides* spp. may explain why the 26G RNAs were not observed within Strongyloididae [49, 52].

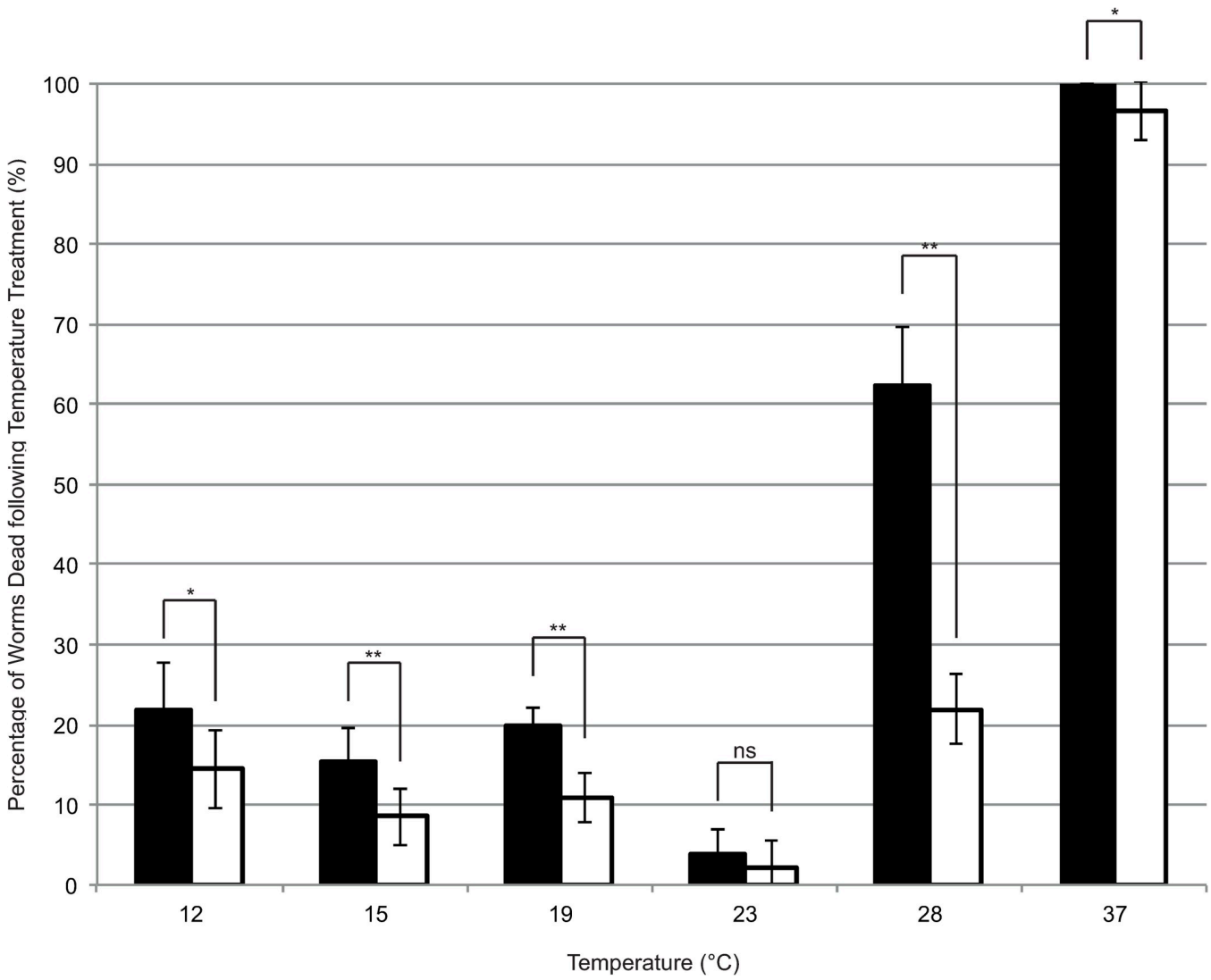


Fig 7. *daf-12(RNAi)* worms (black) have decreased tolerance to temperature stress compared to control worms (white). L1 larvae were soaked with a scrambled siRNA (control) or one targeting *daf-12 (daf-12(RNAi))* and were then exposed to different temperatures for 4 hours, after which their survival was determined. Worms maintained at 12 and 37˚C both had a statistically significant increase in death following temperature stress ($p = 0.003$ and $p = 0.005$). Worms maintained at 15, 19 and 28˚C had a highly statistically significant increase in death following temperature stress (all $p<0.001$) whereas worms maintained at 23˚C had no statistically significant difference in death ($p = 0.083$). Error bars represent standard deviation. The bars represent the mean percentage of worms dead post-heat shock, calculated from three biological replicates, each containing four technical replicates. Mann Whitney U analysis was performed to determine statistical significance. ns indicates a non-statistically significant difference (p-value >0.01), * indicates a statistically significant difference (p-value ≤0.01 - ≥0.001), ** indicates a highly statistically significant difference (p-value ≤0.001).

https://doi.org/10.1371/journal.ppat.1007705.g007

Based on protocols described for *B. malayi*, we developed RNAi for *S. ratti* and demonstrated that it works for three different genes and across several different life stages. Given that three separate genes have been successfully suppressed with minimal off-target effects, we are convinced that with this we can add a robust procedure for gene knock down to the current toolbox in *S. ratti*. Although this toolbox has recently grown considerably [34, 35] we think our addition is substantial. Obviously true mutations (for example those generated by CRISPR/Cas9) do have advantages and are in many cases desirable. However, having the option of knocking down gene functions by RNAi is very useful, in particular in *S. ratti*, where CRISPR/Cas9 works much less efficiently than in *S. stercoralis* (35). *S. ratti* is an important model species,

which, contrary to *S. stercoralis*, can be maintained easily in the laboratory in a natural rodent host. Further, every mutant line must be maintained and this, for an obligatory parasite like *S. ratti*, requires a substantial number of laboratory animals and is laborious and expensive. We showed that our protocol allows the analysis of gene functions at different stages of the part of the life cycle that occurs outside of the host without the need of a host passage. How long the RNAi effect persists in larvae after the infection of a host remains to be determined.

Phenotypic analysis of *daf-12(RNAi)* in *S. ratti*

daf-12 knockdown severely inhibited the formation of L3i of the direct cycle as has been described for DA in *S. papillosus* [5] and *S. stercoralis* [27]. However, the suppression of *daf-12* did not cause the progeny of the free-living generation to form a second free-living generation as it had been observed upon DA addition in *S. papillosus* [5]. Our results are more comparable with the DA results in *S. stercoralis* where the switch of the developmental trajectory was less complete. We found, that *daf-12(RNAi)* progeny of the free-living generation develop slower and in part never complete their differentiation to L3i and those that do reach this stage, although still capable of infecting a host, cause less productive infections. These findings may, but not necessarily do, reflect a partial redirection of development towards non-parasitic L3. It is likely that it is much more difficult to redirect progeny of the free-living generation, which normally all develop into L3i, towards free-living than the progeny of the parasitic generation for which the switch between the two life cycles is normal. As expected, we found *Sra-daf-12* to be involved in the switch between aerobic and anaerobic metabolism. It should be noticed that the effect of knocking down *daf-12* was the same as DA treatment as far as the direct—indirect life cycle switch is concerned but the opposite with respect to the control of genes associated with aerobic to anaerobic metabolism. This confirms that the promotion of the dauer/L3i development is a function of the ligand free- DAF-12 and is inhibited by ligand binding and hence, exogenous DA [5, 27] or depletion of DAF-12 lead to the absence of L3i. On the other hand, promoting aerobic fat usage is achieved by the ligand bound receptor and is therefore enhanced by DA addition [16] but inhibited by *daf-12(RNAi)*. The loss of thermotolerance is also of high importance as these larvae are required by their life cycle to survive and reproduce within the wild at a wide variety of temperatures.

With the RNAi protocol presented, we added a new tool to study gene function in *S. ratti*. Using this tool we showed that *daf-12* is an important gene in *S. ratti* for the control of essentially the same various developmental and metabolic processes as in *C. elegans*. Our results are in full agreement with the hypothesis that the pharmacological effects caused by DA in *Strongyloides* spp. described before [5, 16, 26, 27] involved DAF-12, as suspected but not demonstrated by the authors of these publications and argue for a highly conserved function of *daf-12* in various, only distantly related nematodes. This supports that indeed *daf-12* is a viable target for the development of new target specific antihelminthic drugs. The experiments with DA [23, 27] showed that it is possible to change the developmental pathway of *Strongyloide*s spp. larvae away from differentiating into infective larvae by the administration of small organic molecules (dafachronic acid) both, in and outside of the host. Based on the knowledge of the structure of *S. stercoralis* DAF-12, other inhibitory molecules with optimized pharmacological properties can now be developed.

Materials and methods

Ethics statement

Animal care and use adhered to the German Animal Protection Law (Tierschutzgesetz), the German Animal Protection Laboratory Animal Ordinance (Tierschutz-Versuchstierverordnung) and

EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The procedures for animal maintenance and experiments were ethically and administratively approved by the local govermental authorities in charge (Regierungspräsidium Tübingen), who also issued the necessary permits (AZ35/9185.82–5). The animals were kept in an in-house facility, which is regularly inspected by the local veterinary authorities (Veterinäramt Tübingen). No experiments on human subjects were conducted in this study.

Maintenance of *Strongyloides ratti*

The laboratory strain of *Strongyloides ratti* strain ED321 [58] was used for these experiments. *S. ratti* was kept in our in-house animal facility in female Wistar rats (Charles River Inc). Four week old female rats were injected subcutaneously with around 800 infective larvae each. 7 days post-infection, their feces was collected by placing the infected animals overnight in a cage with a metal grid bottom, lined with wetted paper at 23˚C. The following morning, the feces were collected and cultured in watch glasses in an incubator at 19˚C as described in [59]. 2 days post-collection, the feces were removed from the incubator and the worms were isolated from the cultures using a Baermann funnel as described in [60]. Briefly, fecal materials was wrapped in Linsoft paper and placed in a funnel closed at the bottom with a clamp and filled with tap water, and placed back into the incubator for 2 hours. Following this, the worms that had accumulated at the bottom were removed by briefly opening the clamp and then cleaned. For the experiments that required the use of first or second stage larvae, feces were used within 12 hours of being collected and the worms then isolated the same way.

BLAST analysis of the *Strongyloides* spp. RNAi pathway

Proteins known to be involved in RNAi in *Caenorhabditis elegans* [48] were retrieved from WormBase Parasite (wormbase.org: Version WBPS11) and used as queries for protein BLASTs (BLASTp) and translated nucleotide BLASTs (tBLASTn) against the *Strongyloides* genomes currently listed on WormBase Parasite (parasitewormbase.org: release WBPS11 (WS265): *Strongyloides ratti* (PRJEB125), *Strongyloides papillosus* (PRJEB525), *Strongyloides stercoralis* (PRJEB528), *Strongyloides venezuelensis* (PRJEB530)). To determine whether a protein was present in each Strongyloididae genome, a similar strategy to that in [48] was implemented. After aligning with BLASTp and tBLASTn against the protein of interest, only proteins with an E-value of less than 0.0001 were carried forward. The protein was then searched for in the *C. elegans* genome using tBLASTn and for proteins with a minimal alignment score of 40 bits and an E-value of less than 0.0001, domain structure was analyzed using InterProScan (https://www.ebi.ac.uk/interpro/). If a protein had the same domains as the original *C. elegans* protein, then it was considered to be the reciprocal protein. The full list of proteins in *Strongyloides* genomes can be found in S1 Table and their sequences can be found in S1 Fig.

Identification of the *daf-12* homolog in *S. ratti* and other species of *Strongyloides*

The *daf-12* homolog in *S. ratti* (SRAE_0000032100) was identified through protein BLAST (BLASTp) and translated nucleotide BLAST (tBLASTn) against the whole *C. elegans daf-12* protein and DNA-binding domain (DBD) specifically. All genes with a e-value of 0.0001 were carried forward and reverse searched for in the *C. elegans* genome by tBLASTn. Only those which produced *daf-12* as a likely hit were advanced forward and their domains were then determined using InterProScan. Only genes which contained the same domains as the *C. elegans daf-12* were carried forward. This revealed a single gene (SRAE_0000032100), which is

considered to be the 1 to 1 ortholog of *daf-12* within *S. ratti*. The *daf-12* homologs of *S. stercoralis*, *S. papillosus* and *S. venezuelensis* were identified following the same strategy.

siRNA design and synthesis

Each exon of Sr-*daf-12* was examined both manually and with the siDESIGN center (Dharmacon). Only sequences with a level of predicted on-target activity (>85%) and with no required modifications were considered. All potential sequences were then searched within the *S. ratti* genome using both nucleotide BLAST (BLASTn) and translated nucleotide BLAST (BLASTx) under normal parameters. Sequences with multiple alignments (>3 alignments) greater than 12 amino acids in length were discarded. The remain sequences with the highest predicted on-target activity were then ordered from and synthesized by Eurofins Genomics using the siRNAmax system. This system involves the use of a 19 nucleotide duplex with a 2 UU overhang on the 5' end of both the sense and antisense strands. The list of siRNAs ordered can be found in Table 2.

"Early-stage" Soaking of *S. ratti* with siRNAs

For "Early-stage" soaking experiments, L1 larvae were isolated from fecal culture by Baermann funnel and cleaned repeatedly with water. Worms were then transferred into RNAi culture medium (DMEM (Gibco), Octopamine 20mM (Sigma Aldrich), RNase OUT (Invitrogen) 40U/100μl) in a sterile Eppendorf tube, to which 10mM siRNA was added. The tubes were then incubated at 19˚C for upto 3 days, following which the tubes were removed from the Incubator and briefly centrifuged at 1000g for 3 minutes to collect the worms at the bottom of the tube. The worms were then transferred onto a dry V12 agar plate [60] without any bacteria and examined for any visual phenotypic changes. Half the larvae were transferred into TRIzol (ThermoFisher) for RNA extraction, whilst the other half remained on the plate for use in further experiments.

"Late-stage" Soaking of S. ratti with siRNAs

For the majority of experiments, 2 day old larvae (containing a mix of L3, L4 and free-living adults) were extracted from fecal culture by Baermann funnel and cleaned repeatedly with water. Worms were then transferred onto dry NGM [61] or V12 agar [60] without any bacteria. A modified protocol from [45] was developed to generate RNAi in S. ratti. Worms were then picked into the RNAi culture medium (DMEM (Gibco), Octopamine 20mM (Sigma Aldrich), RNase OUT (Invitrogen) 40U/100μl) in a clean Eppendorf tube, to which 10mM siRNA was added. The tubes were incubated at 19˚C for upto 4 days, following which the tubes were removed from the Incubator and briefly centrifuged at 1000g for 3 minutes to collect the worms at the bottom of the tube. The worms were then transferred onto a dry V12 agar plate [60] without any bacteria and examined for any visual phenotypic changes. Half the larvae were transferred into TRIzol (ThermoFisher) for RNA extraction, whilst the other half remained on the plate for use in further experiments.

RNA extraction, cDNA Synthesis and qRT-PCR

Nematodes (minimum of 10, maximum of 20) were isolated from plates post-soaking and transferred into TRIzol and instantly frozen in liquid nitrogen. The TRIzol/nematode mix was then frozen and thawed three times (with vortexing between freezing) using liquid nitrogen to ensure the cuticle of the worm was disrupted. After the addition of chloroform and centrifugation, the aqueous layer was transferred into a sterile Eppendorf tube and the RNA extracted

from this layer using the RNA Clean & Concentrator-25 Kit (Zymo Research). RNA concentration and purity was then assessed using NanoDrop and the RNA then frozen at -80˚C until needed. cDNA synthesis was then carried out using qScript XLT (Quantabio) as per the manufacturer's protocol. As this kit has the reverse transcriptase already mixed into the buffer, half the RNA sample was retained for use as a negative control against DNA contamination. Following cDNA synthesis, expression level of *daf-12* along with three reference genes *(tbb-1*, *gpd-2*, *rpl-37* [55]) was determined by qRT-PCR using the Light Cycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 II (Roche) machine. Briefly, 5μl of 2x Buffer was mixed with 1ul of each primer, 1μl of cDNA (diluted 1:10 in 1x TE buffer) and 2μl H_2O and run according to the following protocol: Pre-incubation 95˚C for 5 minutes, Amplification 95˚C for 10 seconds, 57˚C for 30 seconds, 72˚C for 20 seconds, 40 cycles, Melting Curve 95˚C for 5 seconds, 65˚C for 1 minute, 97˚C continuous, Cooling 40˚C for 30 seconds. Amplification efficiency was evaluated for each primer pair by running dilution series in six technical replicates (S2 Table). All efficiencies were very close to the expected doubling per round of PCR which translates into a Ct difference of 3.32 for a 10 fold increase. Given this result and that we only compared the same amplicons in different treatments and made not quantitative comparisons of different genes (amplicons) we found it acceptable to use the $2^{-\Delta\Delta Ct}$ method [62]. The relative expressions and fold changes between treatments were calculated separately for each of the three reference genes. The fold changes shown in the figures are the mean values of the three measurements. The full list of primers used in this study can be found in S3 Table.

Fecundity of soaked larvae

L3 worms were isolated as per the "Late-stage" soaking procedure above. After transferring the worms to a dry V12 agar plate without bacteria [60], females were then picked in groups of 10 onto V12 plates with a lawn of HB101. Every 24 hours for the next 96 hours post-transfer, the number of embryos laid was counted with embryos removed from the plate to avoid being counted twice.

Developmental timing and infectivity of soaked larvae

L3/L4 worms were isolated and treated as per the "Late-stage" soaking procedure above. Following 48 hours of soaking, worms were transferred to a V12 agar plate [60] with a lawn of HB101 bacteria. Every 24 hours, all hatched larvae were examined and the total number of L3i was counted. Whether a larva had become a L3i was based upon morphology and whether the worm appeared to have ceased pumping. The L3i were then picked from the plate and stored in PBS at 19˚C.

To test whether *daf-12(RNAi)* L3i were still able to infect rats, 100 *daf-12(RNAi)* L3i (also generated using the "Late-stage" soaking procedure) were injected subcutaneously into a rat. As a control, 100 scrambled siRNA treated L3i were injected subcutaneously into a separate rat. After 7 days incubation, the rat feces was collected daily overnight and incubated at 19˚C as described above. The infective larvae were then collected from the water surrounding the feces and counted manually.

daf-12 RNAi effect upon direct development within *S. ratti*

L1 larvae were isolated from freshly collected rat feces and cleaned. Worms were then incubated using the "Early-stage" soaking procedure. Following treatment, the worm pellet was spun down in a centrifuge and pipetted onto a dry V12 agar plate without bacteria [60]. The total number of worms that had then undergone direct development to L3i and the number

that had undergone indirect development to free-living adults were counted. Males were discarded from the analysis as it is not possible for them to undergo the direct development cycle.

Measurement of fatty acid storage in *S. ratti*

L3/L4 larvae were isolated from rat feces and isolated using the Baermann funnel technique. Following cleaning with PBS, worms were soaked using the "Late-stage" soaking procedure. Following 48 hours soaking, the infective larvae produced were isolated into PBS and concentrated into a small volume by centrifugation. Once at a volume of 20μL or less, the worm pellet was frozen in liquid nitrogen and then sonicated using a Banderon Sonorex 100RH for 45 minutes. Following sonication, free glycerol and triglyceride levels were determined using the Serum Triglyceride Determination Kit (Sigma) according to the manufacturers protocol. To determine total protein concentration as a standard, protein concentration was calculated using DotBlot [63] against a BSA standard.

Differential expression of Aerobic and Anaerobic Metabolism Genes by qRT-PCR

L3/L4 larvae were isolated from rat feces and isolated using the Baermann funnel technique and then treated using the "Late-stage" soaking procedure. Following 48 hours soaking, the infective larvae produced were isolated into PBS and concentrated into a small volume by centrifugation. The worm pellet was transferred into TRIzol and frozen in liquid nitrogen. RNA extraction, cDNA synthesis and qRT-PCR were performed as explained above. To measure aerobic metabolism, *acs-3* and *acbp-3* expression levels were measured. To measure anaerobic metabolism, *ech-8* and *acox-3* expression levels were measured. As a reference, expression of *tbb-1*, *rpl-37* and *gpd-2* [55] was measured. To determine the difference in expression between daf-12(RNAi) and control larvae, fold change for each reference gene was calculated as according [62]. The mean fold change of these three reference genes was then calculated. To account for DNA contamination, raw RNA was included in the qRT-PCR.

Heat Treatment of soaked larvae

L1 larvae were isolated from rat feces using the Baermann funnel technique and then treated using the "Early-stage" soaking procedure. Following 3 days soaking, the worm pellet was collected by briefly centrifuging and then transferred to a V12 plate [60] with a lawn of HB101. Free-living adults were then picked in groups of 20 onto new plates. Plates were then incubated at either 12, 15, 19, 23, 28 or 37˚C for 4 hours. After 4 hours, the plates were examined and the number of alive larvae counted. For each temperature, 3 technical replicates and 3 biological replicates were carried out.

Data analysis

For all experiments, statistical analysis (student's t-test or Mann-Whitney U) was carried out and figures were generated using Excel and Adobe Illustrator. Which test was used for each experiment is indicated in the corresponding figure legend. Statistical probabilities were considered significant once below 0.01.

Supporting information

S1 Table. Table of genes involved in RNAi in Strongyloididae based known *C. elegans* RNAi protein machinery.
(PDF)

S2 Table. Amplification efficiency calculations used for qRT-PCR.
(DOCX)

S3 Table. List of primer sequences used for qRT-PCR.
(DOCX)

S1 Fig. Protein sequences for known *C. elegans* RNAi proteins present within *Strongyloides ratti, Strongyloides papillosus, Strongyloides stercoralis* and *Strongyloides venezuelensis*.
(PDF)

S2 Fig. Protein sequences for daf-12 within *S. ratti, S. papillosus, S. stercoralis* and *S. venezuelensis*.
(PDF)

S1 File. Data File for all graphical information, means and statistical analysis used in this manuscript.
(XLSX)

Acknowledgments

We thank Dorothee Harbecke for her excellent technical assistance and the Department for Integrative Evolutionary Biology for their helpful discussions.

Author Contributions

Conceptualization: Alex Dulovic, Adrian Streit.

Formal analysis: Alex Dulovic.

Investigation: Alex Dulovic, Adrian Streit.

Methodology: Alex Dulovic, Adrian Streit.

Resources: Adrian Streit.

Supervision: Adrian Streit.

Writing – original draft: Alex Dulovic, Adrian Streit.

Writing – review & editing: Alex Dulovic, Adrian Streit.

References

1. Hu PJ. Dauer. In: https://doi.org/10.1895/wormbook.1.144.1, editor. WormBook, ed The *C. elegans* Research Community, WormBook. http://www.wormbook.org2007.
2. Harvey SC, Gemmill AW, Read AF, Viney ME. The control of morph development in the parasitic nematode *Strongyloides ratti*. Proceedings of the Royal Society of London Series B: Biological Sciences. 2000; 267(1457):2057–63. https://doi.org/10.1098/rspb.2000.1249 PMID: 11416909
3. Yamada M, Matsuda S, Nakazawa M, Arizono N. Species-specific differences in heterogonic development of serially transferred free-living generations of *Strongyloides planiceps* and *Strongyloides stercoralis*. The Journal of parasitology. 1991; 77(4):592–4. Epub 1991/08/01. PMID: 1865267.
4. Crook M. The dauer hypothesis and the evolution of parasitism: 20 years on and still going strong. International Journal for Parasitology. 2014; 44(1):1–8. https://doi.org/10.1016/j.ijpara.2013.08.004 PMID: 24095839
5. Ogawa A, Streit A, Antebi A, Sommer RJ. A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. Current biology: CB. 2009; 19(1):67–71. Epub 2008/12/27. https://doi.org/10.1016/j.cub.2008.11.063 PMID: 19110431; PubMed Central PMCID: PMCPmc2712670.

6. Bisoffi Z, Buonfrate D, Angheben A, Boscolo M, Anselmi M, Marocco S, et al. Randomized clinical trial on ivermectin versus thiabendazole for the treatment of strongyloidiasis. PLoS Negl Trop Dis. 2011; 5 (7):e1254. Epub 2011/08/05. https://doi.org/10.1371/journal.pntd.0001254 PMID: 21814588; PubMed Central PMCID: PMCPMC3144183.
7. Vadlamudi RS, Chi DS, Krishnaswamy G. Intestinal strongyloidiasis and hyperinfection syndrome. Clinical and Molecular Allergy. 2006; 4(1):8. https://doi.org/10.1186/1476-7961-4-8 PMID: 16734908
8. Nutman TB. Human infection with *Strongyloides stercoralis* and other related *Strongyloides* species. Parasitology. 2017; 144(3):263–73. Epub 2016/05/18. https://doi.org/10.1017/S0031182016000834 PMID: 27181117; PubMed Central PMCID: PMCPMC5563389.
9. Keiser PB, Nutman TB. *Strongyloides stercoralis* in the Immunocompromised Population. Clinical microbiology reviews. 2004; 17(1):208–17. Epub 2004/01/17. https://doi.org/10.1128/CMR.17.1.208-217.2004 PMID: 14726461; PubMed Central PMCID: PMCPMC321465.
10. Qu TT, Yang Q, Yu MH, Wang J. A Fatal *Strongyloides Stercoralis* Hyperinfection Syndrome in a Patient With Chronic kidney Disease: A Case Report and Literature Review. Medicine. 2016; 95(19): e3638. Epub 2016/05/14. https://doi.org/10.1097/MD.0000000000003638 PMID: 27175679; PubMed Central PMCID: PMCPMC4902521.
11. Geurden T, Chartier C, Fanke J, di Regalbono AF, Traversa D, von Samson-Himmelstjerna G, et al. Anthelmintic resistance to ivermectin and moxidectin in gastrointestinal nematodes of cattle in Europe. International Journal for Parasitology: Drugs and Drug Resistance. 2015; 5(3):163–71. https://doi.org/10.1016/j.ijpddr.2015.08.001 PMID: 26448902
12. Antebi A, Culotti JG, Hedgecock EM. *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. Development (Cambridge, England). 1998; 125(7):1191–205. Epub 1998/05/09. PMID: 9477318.
13. Antebi A, Yeh WH, Tait D, Hedgecock EM, Riddle DL. *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes & development. 2000; 14(12):1512–27. Epub 2000/06/20. PMID: 10859169; PubMed Central PMCID: PMCPMC316684.
14. Shostak Y, Van Gilst MR, Antebi A, Yamamoto KR. Identification of *C. elegans* DAF-12-binding sites, response elements, and target genes. Genes & development. 2004; 18(20):2529–44. Epub 2004/10/19. https://doi.org/10.1101/gad.1218504 PMID: 15489294; PubMed Central PMCID: PMCPMC529540.
15. Hochbaum D, Zhang Y, Stuckenholz C, Labhart P, Alexiadis V, Martin R, et al. DAF-12 Regulates a Connected Network of Genes to Ensure Robust Developmental Decisions. PLOS Genetics. 2011; 7(7): e1002179. https://doi.org/10.1371/journal.pgen.1002179 PMID: 21814518
16. Wang Z, Stoltzfus J, You Y-j, Ranjit N, Tang H, Xie Y, et al. The Nuclear Receptor DAF-12 Regulates Nutrient Metabolism and Reproductive Growth in Nematodes. PLOS Genetics. 2015; 11(3):e1005027. https://doi.org/10.1371/journal.pgen.1005027 PMID: 25774872
17. Penkov S, Kaptan D, Erkut C, Sarov M, Mende F, Kurzchalia TV. Integration of carbohydrate metabolism and redox state controls dauer larva formation in *Caenorhabditis elegans*. Nature Communications. 2015; 6:8060. https://doi.org/10.1038/ncomms9060 https://www.nature.com/articles/ncomms9060-supplementary-information. PMID: 26290173
18. Matyash V, Entchev EV, Mende F, Wilsch-Bräuninger M, Thiele C, Schmidt AW, et al. Sterol-Derived Hormone(s) Controls Entry into Diapause in *Caenorhabditis elegans* by Consecutive Activation of DAF-12 and DAF-16. PLOS Biology. 2004; 2(10):e280. https://doi.org/10.1371/journal.pbio.0020280 PMID: 15383841
19. Ludewig AH, Kober-Eisermann C, Weitzel C, Bethke A, Neubert K, Gerisch B, et al. A novel nuclear receptor/coregulator complex controls *C. elegans* lipid metabolism, larval development, and aging. Genes & development. 2004; 18(17):2120–33. Epub 2004/08/18. https://doi.org/10.1101/gad.312604 PMID: 15314028; PubMed Central PMCID: PMCPMC515290.
20. Hammell CM, Karp X, Ambros V. A feedback circuit involving *let-7*-family miRNAs and DAF-12 integrates environmental signals and developmental timing in *Caenorhabditis elegans*. Proceedings of the National Academy of Sciences. 2009; 106(44):18668–73. https://doi.org/10.1073/pnas.0908131106 PMID: 19828440
21. Liu F, He C-X, Luo L-J, Zou Q-L, Zhao Y-X, Saini R, et al. Nuclear Hormone Receptor Regulation of MicroRNAs Controls Innate Immune Responses in *C. elegans*. PLOS Pathogens. 2013; 9(8): e1003545. https://doi.org/10.1371/journal.ppat.1003545 PMID: 23990780
22. Fisher AL, Lithgow GJ. The nuclear hormone receptor DAF-12 has opposing effects on *Caenorhabditis elegans* lifespan and regulates genes repressed in multiple long-lived worms. Aging Cell. 2006; 5 (2):127–38. https://doi.org/10.1111/j.1474-9726.2006.00203.x PMID: 16626392
23. Patton JB, Bonne-Annee S, Deckman J, Hess JA, Torigian A, Nolan TJ, et al. Methylprednisolone acetate induces, and Delta7-dafachronic acid suppresses, *Strongyloides stercoralis* hyperinfection in NSG mice. Proc Natl Acad Sci U S A. 2017. https://doi.org/10.1073/pnas.1712235114 PMID: 29203662.

24. Stoltzfus JD, Bart SM, Lok JB. cGMP and NHR signaling co-regulate expression of insulin-like peptides and developmental activation of infective larvae in *Strongyloides stercoralis*. PLoS pathogens. 2014; 10 (7):e1004235. Epub 2014/07/11. https://doi.org/10.1371/journal.ppat.1004235 PMID: 25010340; PubMed Central PMCID: PMC4092141.

25. Stoltzfus JD, Minot S, Berriman M, Nolan TJ, Lok JB. RNAseq analysis of the parasitic nematode *Strongyloides stercoralis* reveals divergent regulation of canonical dauer pathways. PLoS Negl Trop Dis. 2012; 6(10):e1854. Epub 2012/11/13. https://doi.org/10.1371/journal.pntd.0001854 PMID: 23145190; PubMed Central PMCID: PMC3493385.

26. Albarqi MMY, Stoltzfus JD, Pilgrim AA, Nolan TJ, Wang Z, Kliewer SA, et al. Regulation of Life Cycle Checkpoints and Developmental Activation of Infective Larvae in *Strongyloides stercoralis* by Dafachronic Acid. PLOS Pathogens. 2016; 12(1):e1005358. https://doi.org/10.1371/journal.ppat.1005358 PMID: 26727267

27. Wang Z, Zhou XE, Motola DL, Gao X, Suino-Powell K, Conneely A, et al. Identification of the nuclear receptor DAF-12 as a therapeutic target in parasitic nematodes. Proceedings of the National Academy of Sciences. 2009; 106(23):9138–43. https://doi.org/10.1073/pnas.0904064106 PMID: 19497877

28. Viney M. Strongyloides. Parasitology. 2016; 144(3):259–62. Epub 11/21. https://doi.org/10.1017/S0031182016001773 PMID: 27759560

29. Streit A. Genetics: modes of reproduction and genetic analysis. Parasitology. 2016:1–11. Epub 2016/03/24. https://doi.org/10.1017/s0031182016000342 PMID: 27003130.

30. Guo L, Chang Z, Dieterich C, Streit A. A protocol for chemical mutagenesis in *Strongyloides ratti*. Exp Parasitol. 2015; 158:2–7. Epub 2015/03/15. https://doi.org/10.1016/j.exppara.2015.03.001 PMID: 25765558.

31. Viney ME, Green LD, Brooks JA, Grant WN. Chemical mutagenesis of the parasitic nematode *Strongyloides ratti* to isolate ivermectin resistant mutants. International Journal for Parasitology. 2002; 32 (14):1677–82. https://doi.org/10.1016/S0020-7519(02)00157-1. PMID: 12464413

32. Nemetschke L, Eberhardt AG, Hertzberg H, Streit A. Genetics, chromatin diminution, and sex chromosome evolution in the parasitic nematode genus *Strongyloides*. Current biology: CB. 2010; 20 (19):1687–96. Epub 2010/09/14. https://doi.org/10.1016/j.cub.2010.08.014 PMID: 20832309.

33. Lok JB, Shao H, Massey HC, Li X. Transgenesis in *Strongyloides* and related parasitic nematodes: historical perspectives, current functional genomic applications and progress towards gene disruption and editing. Parasitology. 2016; 144(3):327–42. Epub 03/22. https://doi.org/10.1017/S0031182016000391 PMID: 27000743

34. Hunt VL, Tsai IJ, Coghlan A, Reid AJ, Holroyd N, Foth BJ, et al. The genomic basis of parasitism in the *Strongyloides* clade of nematodes. Nat Genet. 2016; 48(3):299–307. Epub 2016/02/02. https://doi.org/10.1038/ng.3495 PMID: 26829753.

35. Gang SS, Castelletto ML, Bryant AS, Yang E, Mancuso N, Lopez JB, et al. Targeted mutagenesis in a human-parasitic nematode. PLOS Pathogens. 2017; 13(10):e1006675. https://doi.org/10.1371/journal.ppat.1006675 PMID: 29016680

36. Viney M, Kikuchi T. *Strongyloides ratti* and *S. venezuelensis*—rodent models of *Strongyloides* infection. Parasitology. 2016:1–10. Epub 2016/03/05. https://doi.org/10.1017/s0031182016000020 PMID: 26935155.

37. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998; 391:806. https://doi.org/10.1038/35888 https://www.nature.com/articles/35888-supplementary-information. PMID: 9486653

38. Tabara H, Grishok A, Mello CC. RNAi in *C. elegans*: soaking in the genome sequence. Science (New York, NY). 1998; 282(5388):430–1. Epub 1998/12/05. PMID: 9841401.

39. Bakhetia M, Charlton W, Atkinson HJ, McPherson MJ. RNA Interference of Dual Oxidase in the Plant Nematode *Meloidogyne incognita*. Molecular Plant-Microbe Interactions. 2005; 18(10):1099–106. https://doi.org/10.1094/MPMI-18-1099 PMID: 16255249

40. Urwin PE, Lilley CJ, Atkinson HJ. Ingestion of Double-Stranded RNA by Preparasitic Juvenile Cyst Nematodes Leads to RNA Interference. Molecular Plant-Microbe Interactions. 2002; 15(8):747–52. https://doi.org/10.1094/MPMI.2002.15.8.747 PMID: 12182331

41. Ratnappan R, Vadnal J, Keaney M, Eleftherianos I, O'Halloran D, Hawdon JM. RNAi-mediated gene knockdown by microinjection in the model entomopathogenic nematode *Heterorhabditis bacteriophora*. Parasites & Vectors. 2016; 9(1):160. https://doi.org/10.1186/s13071-016-1442-4 PMID: 26993791

42. Morris R, Wilson L, Sturrock M, Warnock ND, Carrizo D, Cox D, et al. A neuropeptide modulates sensory perception in the entomopathogenic nematode *Steinernema carpocapsae*. PLOS Pathogens. 2017; 13(3):e1006185. https://doi.org/10.1371/journal.ppat.1006185 PMID: 28253355

43. Blanchard A, Guégnard F, Charvet CL, Crisford A, Courtot E, Sauvé C, et al. Deciphering the molecular determinants of cholinergic anthelmintic sensitivity in nematodes: When novel functional validation approaches highlight major differences between the model *Caenorhabditis elegans* and parasitic species. PLOS Pathogens. 2018; 14(5):e1006996. https://doi.org/10.1371/journal.ppat.1006996 PMID: 29719008

44. McCoy CJ, Warnock ND, Atkinson LE, Atcheson E, Martin RJ, Robertson AP, et al. RNA interference in adult *Ascaris suum*—an opportunity for the development of a functional genomics platform that supports organism-, tissue- and cell-based biology in a nematode parasite. International journal for parasitology. 2015; 45(11):673–8. https://doi.org/10.1016/j.ijpara.2015.05.003 PMID: 26149642.

45. Misra S, Gupta J, Misra-Bhattacharya S. RNA interference mediated knockdown of *Brugia malayi* UDP-Galactopyranose mutase severely affects parasite viability, embryogenesis and in vivo development of infective larvae. Parasites & vectors. 2017; 10(1):34–. https://doi.org/10.1186/s13071-017-1967-1 PMID: 28103957.

46. Viney ME, Thompson FJ. Two hypotheses to explain why RNA interference does not work in animal parasitic nematodes. Int J Parasitol. 2008; 38(1):43–7. Epub 2007/11/22. https://doi.org/10.1016/j.ijpara.2007.08.006 PMID: 18028931.

47. Somarathne M, Gunawardene Y, Chandrasekharan NV, Dassanayake RS. Development of siRNA mediated RNA interference and functional analysis of novel parasitic nematode-specific protein of *Setaria digitata*. Exp Parasitol. 2018; 186:42–9. Epub 2018/02/16. https://doi.org/10.1016/j.exppara.2018.02.001 PMID: 29448039.

48. Dalzell JJ, McVeigh P, Warnock ND, Mitreva M, Bird DM, Abad P, et al. RNAi Effector Diversity in Nematodes. PLOS Neglected Tropical Diseases. 2011; 5(6):e1176. https://doi.org/10.1371/journal.pntd.0001176 PMID: 21666793

49. Holz A, Streit A. Gain and Loss of Small RNA Classes—Characterization of Small RNAs in the Parasitic Nematode Family Strongyloididae. Genome Biology and Evolution. 2017; 9(10):2826–43. https://doi.org/10.1093/gbe/evx197 PMID: 29036592

50. Tijsterman M, May RC, Simmer F, Okihara KL, Plasterk RHA. Genes Required for Systemic RNA Interference in *Caenorhabditis elegans*. Current Biology. 2004; 14(2):111–6. https://doi.org/10.1016/j.cub.2003.12.029. PMID: 14738731

51. Shih JD, Hunter CP. SID-1 is a dsRNA-selective dsRNA-gated channel. RNA (New York, NY). 2011; 17(6):1057–65. Epub 2011/04/09. https://doi.org/10.1261/rna.2596511 PMID: 21474576; PubMed Central PMCID: PMCPMC3096038.

52. Zhang C, Montgomery TA, Gabel HW, Fischer SEJ, Phillips CM, Fahlgren N, et al. *mut-16* and other *mutator* class genes modulate 22G and 26G siRNA pathways in *Caenorhabditis elegans*. Proceedings of the National Academy of Sciences. 2011; 108(4):1201–8. https://doi.org/10.1073/pnas.1018695108 PMID: 21245313

53. Shostak Y, Yamamoto KR. Overlapping but separable determinants of DNA binding and nuclear localization map to the C-terminal end of the *Caenorhabditis elegans* DAF-12 DNA binding domain. The Journal of biological chemistry. 2005; 280(8):6554–60. Epub 2004/12/22. https://doi.org/10.1074/jbc.M412928200 PMID: 15611047.

54. Snow MI, Larsen PL. Structure and expression of *daf-12*: a nuclear hormone receptor with three isoforms that are involved in development and aging in *Caenorhabditis elegans*. Biochimica et Biophysica Acta (BBA)—Gene Structure and Expression. 2000; 1494(1):104–16. https://doi.org/10.1016/S0167-4781(00)00224-4.

55. Biewener V, Welz C, Khumpool G, Küttler U, Schnieder T. *Strongyloides papillosus*: Changes in transcript levels of lysozyme and aspartic protease 2 in percutaneously migrated larvae. Experimental Parasitology. 2012; 132(1):62–8. https://doi.org/10.1016/j.exppara.2012.02.009 PMID: 22366363

56. Miller MA, Ruest PJ, Kosinski M, Hanks SK, Greenstein D. An Eph receptor sperm-sensing control mechanism for oocyte meiotic maturation in *Caenorhabditis elegans*. Genes & development. 2003; 17(2):187–200. https://doi.org/10.1101/gad.1028303 PMID: 12533508.

57. Zhuang JJ, Hunter CP. Tissue Specificity of *Caenorhabditis elegans* Enhanced RNA Interference Mutants. Genetics. 2011; 188(1):235–7. https://doi.org/10.1534/genetics.111.127209 PMID: 21385728

58. Viney ME. Developmental switching in the parasitic nematode *Strongyloides ratti*. Proceedings of the Royal Society of London Series B: Biological Sciences. 1996; 263(1367):201–8. https://doi.org/10.1098/rspb.1996.0032 PMID: 8728983

59. Viney ME, Matthews BE, Walliker D. On the biological and biochemical nature of cloned populations of *Strongyloides ratti*. Journal of helminthology. 1992; 66(1):45–52. Epub 1992/03/01. PMID: 1469259.

60. Dulovic A, Puller V, Streit A. Optimizing culture conditions for free-living stages of the nematode parasite *Strongyloides ratti*. Experimental Parasitology. 2016; 168:25–30. https://doi.org/10.1016/j.exppara.2016.06.005 PMID: 27334397

61. Stiernagle T. Maintenance of C. elegans. WormBook, ed The C. elegans Research Community, Wormbook, 101895/wormbook11011, http://wwwwormbookorg. 2006.

62. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods. 2001; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

63. Serobyan V, Xiao H, Namdeo S, Rödelsperger C, Sieriebriennikov B, Witte H, et al. Chromatin remodelling and antisense-mediated up-regulation of the developmental switch gene *eud-1* control predatory feeding plasticity. Nature Communications. 2016; 7:12337. https://doi.org/10.1038/ncomms12337 https://www.nature.com/articles/ncomms12337-supplementary-information. PMID: 27487725