INFLUENCE OF *XENORHABDUS* SYMBIONTS ON GONAD DEVELOPMENT AND PHEROMONE PRODUCTION OF FIRST-GENERATION ADULT *STEINERNEMA* NEMATODES (NEMATODA: STEINERNEMATIDAE)

by

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STATEMENT BY AUTHOR

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
EPNs	Entomopathogenic nematodes
IJs	Infective juveniles
LB	Luria-Bertani
NBTA	Nutrient agar supplemented with 0.0025% (w/v) bromothymol blue, 0.004% (w/v) triphenyltetrazolium, and 0.1% (w/v) sodium pyruvate
XnAll	*Xenorhabdus nematophila* symbiont associated with *S. carpocapsae* All strain
XnAna	*Xenorhabdus nematophila* symbiont associated with *S. anatoliense*
XbfSN	*Xenorhabdus bovienii* symbiont associated with *S. feltiae* SN strain
Xbp	*Xenorhabdus bovienii* symbiont associated with *S. puntauvense* Li6 strain
TBL	Total body length
MBW	Maximum body width
AE-G	Anterior-end to beginning of gonad
TREF	Testis reflexion length
TGL	Total gonad length
AGL	Anterior gonad length
PGL	Posterior gonad length
G	Ratio of total gonad length to total body length (total gonad length/total body length)

ABSTRACT

Entomopathogenic *Steinernema* nematodes (Nematoda: Steinernematidae) have a mutualistic relationship with *Xenorhabdus* bacteria (Gamma-Proteobacteria Enterobacteriaceae). The two partners form an insecticidal alliance that is successful in killing a wide range of insects. A few studies have shown that *Steinernema* IJs have an enhanced virulence and reproductive fitness when they associate with their cognate symbionts. However, there are unanswered questions regarding the physiological interactions that govern and perpetuate the interactions between different nematode developmental stages and their bacterial partners. In this study, we evaluated gonad development and maturation time of first-generation adults of *S. carpocapsae* and *S. feltiae* adults when reared under four bacterial scenarios: a) cognate symbiotic, b) non-cognate symbiotic bacterial strain, c) non-cognate symbiotic bacterial species and d) non-symbiotic bacteria *(Serratia proteamaculans*). For comparative purposes, we also considered adult nematodes reared *in vivo* in *Galleria mellonella* larvae to assess nematode development under natural conditions. Furthermore, in this study we also measured production of nematode pheromones (ascarosides), which play a key role in mating and reproduction. For this purpose, we considered *in vitro* rearing methods (with cognate and non-cognate *Xenorhabdus* symbionts) to qualitatively and quantitatively characterize ascarosides produced by first-generation adults. Our data showed that for both *Steinernema* spp. tested, time to adult maturation and gonad development was tightly dependent on the bacterial conditions under which juveniles were reared. However, contrasting results were observed when assessing total body length and gonad size. *S. feltiae* males and females size (body length and width) and respective gonad length were smaller when reared with a non-cognate symbiotic species. Additionally, non-symbiotic bacteria did not sustain *S. feltiae* maturation to adult stages. Contrarily, *S. carpocapsae* juveniles

developed to adults when reared with any of the bacterial conditions tested, including with non-symbiotic *Serratia proteamaculans*. Additionally, *S. carpocapsae* adults, unlike *S. feltiae*, did not exhibit enhanced body and gonad size when reared with their cognate symbiont. In fact, *S. carpocapsae* males and females had larger gonad lengths when reared with a non-cognate symbiotic strain, XnAna (*X. nematophila* associated with *S. anatoliense*). *S. carpocapsae* males and females had significantly underdeveloped gonads when reared with non-symbiotic bacteria. In both *Steinernema* spp., sex ratio was not impacted by the bacterial condition. However, sex ratio (female: male) *S. carpocapsae,* decreased from 2:1 to 1:1 when reared with non-symbiotic bacteria. The body and gonad sizes of *Steinernema* spp. reared *in vitro* with their cognate symbiont were significantly smaller than those grown *in vivo*. Ascaroside production in either *Steinernema* spp. was not significantly impacted by the rearing conditions. In *S. carpocapsae*, a significant increase in glucoside-1 was observed when the nematodes were reared with cognate or non-cognate bacteria. No detectable quantities of asc-C11 were produced by *S. feltiae* nematodes when reared with a non-cognate symbiotic bacterial species. We conclude that bacterial symbionts influenced maturation and development of first-generation adults' in both *Steinernema* spp. tested in this study. However, response to the bacterial symbionts was species specific. Additionally, this study showed that *Xenorhabdus* as a food source plays an important role in the type and amount of ascarosides produced by *Steinernema* spp.

CHAPTER 1: LITERATURE REVIEW

The phylum Nematoda

Nematoda Cobb, 1932 is a species-rich animal phylum that is estimated to encompass 500,000-1,000,000 species (Lambshed and Boucher, 2003). Nematodes are ubiquitous and abundant organisms on Earth due to their ability to exist in almost every possible habitat and ecosystem (Bernard, 1992; Ettema, 1998; De Ley, 2006; Stock, 2015). In fact, four out of every five multicellular animals on the planet are nematodes (Platt, 1994; Blaxter and Bird, 1997). These organisms exist in a variety of niches, varying from aquatic environments (marine and fresh water) to terrestrial environments, playing an important role in the ecosystem (Yeates, 1987; Lambshed and Boucher, 2003).

Nematodes have developed a wide range of lifestyles, spanning from commensalistic and fortuitous associations, to highly specialized parasitic interactions with plants and animals (Bedding, 1967; Peterson, 1985; Blaxter and Bird, 1997; Ettema, 1998; Knopp et al., 2012). In particular, parasitic species have received a lot more attention than free-living taxa due to the damage they cause to crops, livestock, and humans (Wallace, 1963; Poinar 1983; Anderson, 2000; Stock, 2015; Weinstein and Lafferty, 2015). For example, over 100 species of nematodes cause disease in humans, and over 5,000 species are known to infect wildlife animals (Weinstein and Lafferty, 2015).

However, certain parasitic species can be used to benefit humans. This is the case of the so called entomopathogenic nematodes (hereafter referred as EPNs), which comprise two families, Steinernematidae Travassos, 1927 and Heterorhabditidae Poinar, 1975. These nematodes have an obligate mutualistic association with Enterobacteriaceae bacteria (γ-Proteobacteria) that is necessary for the completion of their lifecycle (Poinar, 1990; Boemare et

al., 2002). EPNs in the genus *Steinernema* (Steinernematidae) associate with *Xenorhabdus* Thomas and Poinar, 1979 bacteria, whereas *Heterorhabditis* (Heterorhabditidae) nematodes associate with *Photorhabdus* Boemare, Akhurst and Mourant, 1993 bacteria (Boemare et al., 1993; Boemare 2002).

For several decades, EPNs have been studied and utilized as control agents of insect-pests in augmentative, conservational and classical biological control programs (Gaugler and Kaya, 1993; Grewal et al., 2005; Lacey and Georgis, 2012). However, over the past two decades, research has spurred beyond pest management and agricultural studies (Stock and Goodrich-Blair, 2008; Campos-Herrera et al., 2012; Stock 2015). Indeed, EPNs and their bacterial symbionts are now viewed as model systems for studying physiological, chemical and development aspects regarding mutualistic symbiotic associations (Stock, 2005; Stock and Goodrich-Blair, 2008; Eleftherianos et al., 2010; Dillman et al., 2012).

In this study, the focus was on *Steinernema* nematodes and their *Xenorhabdus* symbionts. Therefore, the following sections will specifically address this mutualistic partnership, placing emphasis on the hallmarks of their symbiotic interactions.

***Steinernema-Xenorhabdus* lifecycle**

As previously stated, *Steinernema* nematodes have an obligate mutualistic association with *Xenorhabdus* bacteria (Boemare et al., 1993; Boemare, 2002). These bacteria cannot live freely in the soil, and thus are vectored by the *Steinernema* infective juvenile (IJs) nematodes from one insect host to another (Gaugler and Kaya, 1993). IJs are third-stage juveniles that resemble the dauer-stage of *Caenorhabditis elegans* nematodes, which is also a non-feeding and immature stage. *Steinernema* IJs are unique in that they harbor their *Xenorhabdus* bacterial

symbionts in a specialized receptacle in the anterior portion of the intestine known as the "bacterial receptacle" (Bird and Akhurst, 1983; Snyder et al., 2007). Once a suitable insect host is found, IJs enter the insect host via natural orifices (e.g. mouth, spiracles, and anus). IJs may travel through the insect's digestive or respiratory system and migrate to the hemocoel.

Upon contact with the insect's hemolymph (i.e. blood and lymph), the IJs exsheath their outer cuticle, and transition to a "recovering phase." During this phase, the IJ's intestine initiates peristaltic movements, which facilitate the release of *Xenorhabdus* via the anus (Snyder et al. 2007). *Xenorhabdus* then utilize available nutrients in the insect host to multiply and produce an arsenal of toxins and secondary metabolites that cause a massive septicemia, which kills the insect host in 24 to 48 hours (Boemare et al. 1993; Boemare, 2002). The toxins and secondary metabolites produced by *Xenorhabdus* lead to the degradation of insect tissue, providing nutrients for the nematodes to grow. The IJs molt to the fourth juvenile stage, which is followed by one last molt that leads to their adulthood (Fig. 1).

Steinernema adults reproduce in a natural monoxenic condition, with *Xenorhabdus* as their only food source (Boemare et al., 2002). In this respect, several studies have reported that *Xenorhabdus* produce a wide array of antimicrobial molecules, which kill the insect's natural microflora and prevent the invasion of the host cadaver from saprobic microbes (Akhurst, 1982; Chen et al., 1994).

Most *Steinernema* species are amphimictic, with distinct male and female stages in all adult generations that develop inside an insect host. However, one species, *Steinernema hermaphroditum* is so far the only species know to have hermaphroditic females and no, or scant, males in the first adult generation (Griffin et al., 2001; Stock et al., 2004). Mature first-generation adults copulate, and fertilized females lay eggs from which first-stage juveniles' hatch.

Juveniles molt four times until they become adults. Usually there are two adult generations in an insect cadaver depending on host size and food availability. First-generation adults and subsequent adult generations are morphologically similar, except first-generation adults are much larger than subsequent adult generations (Nguyen et al. 2007).

The *Steinernema-Xenorhabdus* life cycle continues in the insect cadaver until food resources become scarce. Additionally, accumulation of certain chemical cues, such as carbon dioxide and ammonia, inside the insect cadaver triggers IJ formation and their emergence from the insect cadaver (San-Blas et al., 2008). Martens et al. (2003) showed that in *S. carpocapsae* IJs only a few *Xenorhabdus* cells (1-3) colonize their receptacles before they emerge from the insect cadaver.

Specificity of the *Xenorhabdus-Steinernema* partnership

A few studies have provided evidence of the stringent association and the reciprocal level of dependence between *Xenorhabdus* and *Steinernema* for their survival, virulence and reproduction (Poinar and Thomas, 1966; Sicard et al., 2004; Murfin et al., 2015; McMullen et al. 2017). As mentioned previously, *Steinernema* nematodes house *Xenorhabdus* symbionts in a specialized receptacle and play a key role in their dissemination from one insect host to another (Snyder et al., 2007). In return, *Xenorhabdus* provide nutrients necessary for the nematodes to grow and develop, mature, and reproduce. *Xenorhabdus* also contribute to the creation of a safe environment in the insect cadaver, which prevents saprobic microbes and other soil predators from potentially interrupting the nematode life cycle (Poinar and Thomas, 1966; Akhurst, 1982; Chen et al., 1994).

Each *Steinernema* nematode associates with one specific *Xenorhabdus* species; however, a given *Xenorhabdus* sp. may associate with more than one *Steinernema* (Fischer-Le Saux et al., 1999; Boemare, 2002). In relation to this, Lee and Stock (2010) were the first to propose a phylogenetic hypothesis for co-evolution between these two partners considering a multigene approach. The study concluded that in spite of the obligate nature of the symbiosis, there is no perfect congruence between the evolutionary histories of the nematodes and their bacterial symbionts. For example, out of the 30 pairs studied, there were only 12 co-speciation events, and 17 host switching events (Lee and Stock, 2010). The lack of perfect co-cladogenesis may be the result of co-infection events followed by the displacement of a given *Xenorhabdus* species and the horizontal acquisition of a new symbiotic partner (Lee and Stock, 2010).

At the genetic level, the *nilABC* (nematode intestinal localization) locus present in the *X. nematophila* genome plays an important role in the colonization of the IJ receptacle in *S. carpocapsae* (Heungens et al., 2002; Cowles and Goodrich-Blair, 2008). Specifically, the insertion of *nilABC* into other *Xenorhabdus* species increases the ability of non-cognate *Xenorhabdus* species to colonize *Steinernema carpocapsae* (Cowles and Goodrich-Blair, 2008). However, Chapuis et al. (2009) demonstrated in the laboratory that one heterospecific symbiont *Xenorhabdus griffinae* can colonize *S. carpocapsae*. Until now, this is the only known non-cognate *Xenorhabdus* sp. that is able to colonize this nematode species experimentally. The presence of *nilABC* in this species has not yet been determined, but this finding may indicate that this bacterium has this locus and may also be involved with its nematode host interactions.

Role of *Xenorhabdus* symbionts on *Steinernema* fitness

As in many mutualistic symbioses, the relationship between *Steinernema* and *Xenorhabdus* is tightly maintained due to the benefits the two interacting partners exchange with each other. For example, virulence and reproduction of nematodes inside an insect host are linked, and even enhanced, by the presence of specific bacterial symbionts (Poinar and Thomas, 1966; Sicard et al., 2003; Sicard et al., 2004; Chapuis et al., 2012; Murfin et al., 2015; McMullen et al., 2017).

Steinernema virulence and reproduction are greatly impacted when the nematodes are disassociated from their cognate symbionts and are paired with non-cognate symbionts (Sicard et al., 2004; Murfin et al., 2015; McMullen et al., 2017). For example, Sicard et al. (2004) assessed the specificity between *S. carpocapsae* and its native *X. nematophila*. They observed that *S. carpocapsae* exhibited lower reproductive rates and were less virulent when associated with non-cognate symbionts. When nematodes were associated with non-cognate symbionts, they failed to transmit the symbionts to their offspring (Sicard et al., 2004).

Similarly, Murfin et al. (2015) reported strain (subspecies) specificity in certain *Steinernema-Xenorhabdus* pairs. In their study, the authors showed that *S. feltiae* fitness is impacted when the nematodes associate with *X. bovienii* isolates from different *S. feltiae* strains.

More recently, McMullen et al. (2017) assessed the role of cognate and non-cognate *X. bovienii* symbionts on the virulence and reproductive fitness of three different *Steinernema* hosts. For example, when *Steinernema* nematodes associated with non-cognate symbionts, they were less virulent, and had lower progeny production than cognate pairings (McMullen et al., 2017).

Altogether, these studies demonstrate that *Steinernema* may associate with different *Xenorhabdus* strains, but there are fitness costs in relation to the bacterial species/strain with

which they associate.

Xenorhabdus also play an important role on *Steinernema* development. An early study by Poinar and Thomas (1966) reported that when DD-136 (*Neoaplectana* sp. Steinernematidae) nematodes were reared with non-symbiotic bacteria, or without bacteria, the nematodes were unable to reproduce. The authors also noted, that mating attempts decreased and reproductive organs “appeared degenerate” when bacteria was not present, suggesting that bacterial symbionts support gonad development. However, no supporting data sustained this claim, nor were further studies carried out.

The role of bacterial symbionts on development and fertility has been observed in other nematode groups. For example, a few studies have shown that fertility in filarial nematodes decreased when their *Wolbachia* endosymbionts were depleted (Bosshardt et al., 1993; Hoerauf et al., 1999; Hoerauf et al., 2000; Taylor et al., 2005). Specifically, Hoerauf et al. (1999) demonstrated that the removal of *Wolbachia* with the antibiotic tetracycline caused infertility and inhibition of embryogenesis in female nematodes. Rao and Well (2002) also reported similar findings in the filarial worm *Brugia malayi*.

Although it has been shown that *Xenorhabdus* symbionts affect *Steinernema* reproductive fitness, no studies have been conducted to specifically assess how the bacteria contributes to adult development and gonad maturation. In this respect, the present study will investigate the effect of symbiotic (cognate and non-cognate) and non-symbiotic bacteria have on gonad development, maturation time and sex ratio of first-generation *Steinernema* adults.

Nematode Ascarosides

Ascarosides are signaling molecules that were first discovered in the human parasite *Ascaris lumbricoides* (Ascaridia) (Flury, 1912). The ascarosides obtained from this parasite were highly lipophilic and formed an internal coating in *Ascaris* eggs (Jezyk and Fairbairn, 1967; Bartley et al., 1996). This internal coating protected the eggs from harsh environmental and chemical conditions (Fairbairn, 1957; Foor, 1967). It is now known that these molecules consist of a dideoxysugar ascarylose sugar base, and fatty acid side chains (Ludewig and Schroeder, 2013).

Ascarosides have been widely studied in *Caenorhabditis elegans* where they act as pheromones, and influence nematode behavior and development (Golden and Riddle, 1982; Golden and Riddle, 1984; Jeong et al., 2005; Butcher et al., 2007; Srinivasan et al., 2008; Kaplan et al., 2011; Srinivasan et al. 2012). These signal molecules are present across the phylum Nematoda, but the "cocktail" that each species produces is unique, varying qualitatively and quantitatively (Choe et al., 2012a).

A few nomenclature systems exist for ascarosides. The remainder of this work utilizes the structure-based nomenclature of ascarosides (e.g. asc-C6-MK), rather than the nomenclature used for their discovery order (e.g. ascr#1) or the carbon length nomenclature (e.g. C5).

Golden and Riddle (1982) were the first to demonstrate that ascarosides influence development in *C. elegans*. Ascarosides increased dauer formation and inhibited their recovery, even in the presence of abundant food (which promotes dauer recovery). The molecular structure of the dauer-inducing pheromone was first reported by Jeong et al. (2005) and later described as "daumone" (asc-C7). Further research showed that dauer-formation is not solely due to one single ascaroside, but to synergistic interactions of several ascaroside molecules

(Butcher et al., 2007). Specifically, Butcher at al. (2007) demonstrated that two other molecules, asc-C6-MK and asc-ΔC9 are components of the dauer-inducing pheromone. Butcher et al. (2008) also discovered another ascaroside molecule, asc-ωC3, which acts synergistically with the other dauer ascarosides (Butcher et al., 2008). Additionally, IC-asc-C5, an indole ascaroside derivative, is involved in dauer formation, and has unique characteristics in response to concentration (Butcher at al., 2009). Specifically, at high concentrations this molecule inhibits dauer formation (Butcher et al., 2009). Pungaliya et al. (2009) discovered that another ascaroside, asc-ΔC7-PABA, is a component of the dauer pheromone.

Besides developmental regulation, ascarosides modulate male attraction. For example, Simon and Sternberg (2002) demonstrated that *C. elegans* hermaphrodites produce a chemical cue used to signal males. Srinivasan et al. (2008) elucidated the blend of ascarosides that mediates this attraction. Specifically, the authors showed that a synergistic blend of three molecules: asc-C6-MK, asc-ΔC9 and Glc-asc-C6-MK functions as a potent male attractant at low concentrations. Interestingly, at higher concentrations, the blend has a deterrent effect on hermaphrodites, and does not attract males (Srinivasan et al., 2008). This study also demonstrated that variation of ascaroside blends (quali- and quantitative) trigger different behaviors. Pungaliya et al. (2009) discovered that another ascaroside, asc-ΔC7-PABA, functions as a potent male attractant.

von Reuss et al. (2012) showed that indole ascarosides are less common than their non-indole ascarosides. Although not commonly produced, these signals play a key role in nematode aggregation and promote attraction of both solitary and social behaviors in *C. elegans* (Srinivasan et al., 2012). Srinivasan et al. (2012) demonstrated that asc-ΔC9 repulses hermaphrodites, whereas tryptophan-derived indole ascarosides attracts them.

Izrayelit et al. (2010) demonstrated that ascaroside biosynthesis is also sex specific. In relation to this, Choe et al. (2012b) found that asc-C7 produced by *Panagrellus redivivus* females strongly attracts males. Additionally, a novel dihydroxy derivative of asc-C11 produced by males also attracts females (Choe et al., 2012b).

Several studies have demonstrated that ascaroside signaling is dependent on bacterial food supply. Golden and Riddle (1982, 1984) demonstrated that in *C. elegans*, bacterial food supply acts competitively with the dauer pheromone to control development. The dauer pheromone increases dauer formation and is released in response to low food supply (Golden and Riddle, 1982). Food, contrastingly, serves as a signal that enhances the recovery of nematodes from the dauer stage (Golden and Riddle, 1984). Additionally, a study by Kaplan et al. (2011) demonstrated that when *C. elegans* nematodes were reared with an abundance of *Escherichia coli*, more ascarosides were detectable and quantifiable when compared to *C. elegans* reared in a starvation condition.

With respect to EPNs, a study by Choe et al. (2012a) reported six molecules present in *S. carpocapsa*e, including: asc-C4, asc-C5, asc-C6, asc-C7, asc-C9, and asc-C11 (Choe et al., 2012a). Kaplan et al. (2012), demonstrated that asc-C5 is universal among EPNs, while ascaroside asc-C4 is apparently specific to *Steinernema* species.

A few studies have also shown that ascarosides play a role in IJ formation of both EPN genera, *Steinernema* (Kaplan et al., 2012) and *Heterorhabditis* (Noguez et al., 2012). Additionally, IJ dispersal from the insect cadaver in *Steinernema* is mediated by ascaroside signaling (Kaplan et al., 2012).

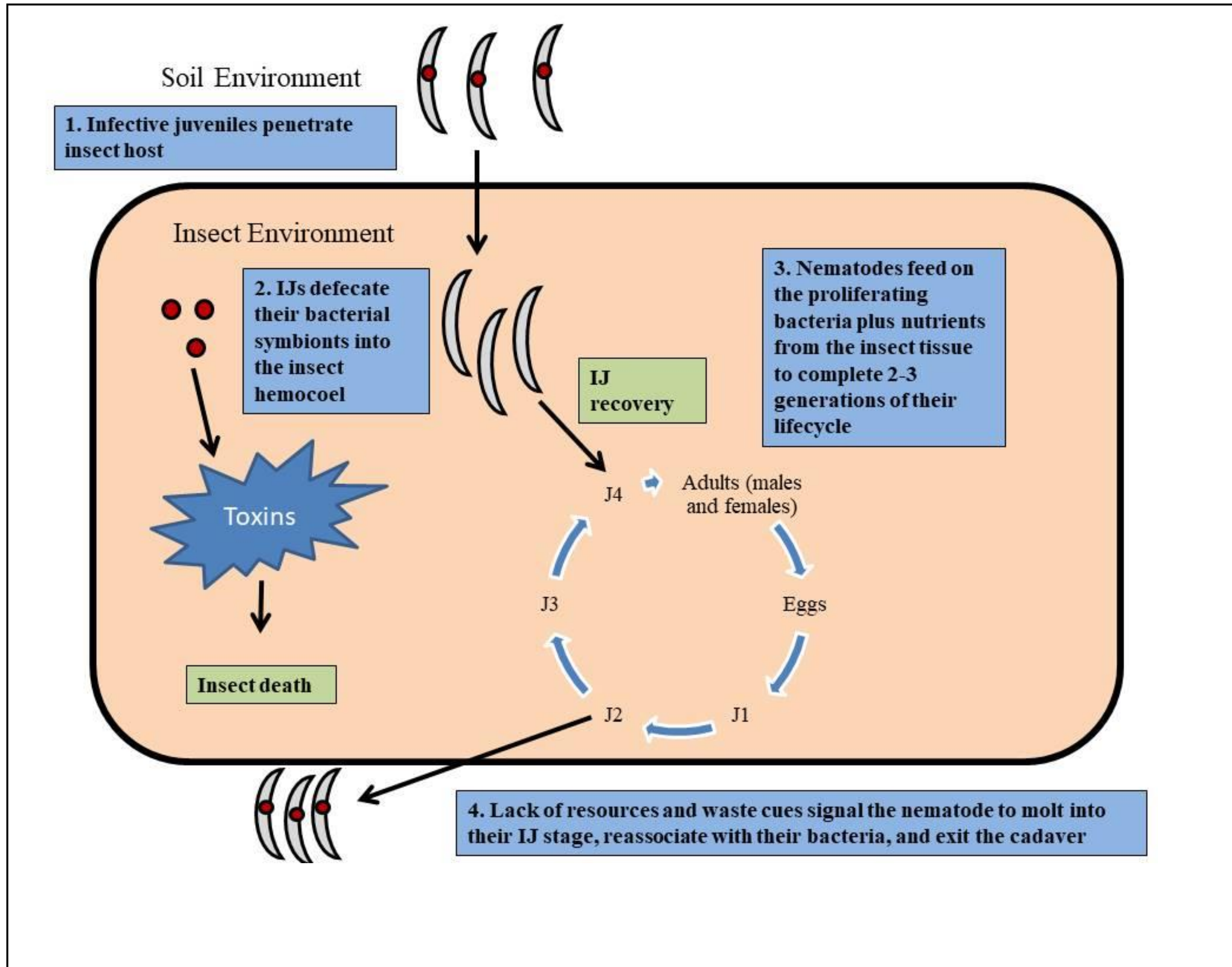
As mentioned before, EPNs have an obligate mutualistic association with *Xenorhabdus* bacteria (Boemare et al., 1993; Boemare, 2002). *Xenorhabdus* symbionts facilitate many aspects

of the *Steinernema* life cycle, including: a) acting as a food source, thus contributing to development in the insect cadaver, b) allowing the IJs to recover and continue their growth and reproduction. Disruption of this partnership hampers the fitness of *Steinernema* nematodes (Sicard et al., 2003; Sicard et al., 2004; Murfin et al., 2015; McMullen et al., 2017). However, until now, no research has focused on elucidating the role that *Xenorhabdus*, as a food source, may play on ascaroside production in *Steinernema* nematodes. In this respect, the present study will evaluate the role of cognate and non-cognate symbiotic bacteria on ascaroside production in first-generation *Steinernema* adults.

Table 1. Ascaroside structure-based nomenclature and their discovery order nomenclature synonym

Structure-based nomenclature	***Discovery order synonym***
asc-C7	ascr#1
asc-C6-MK	ascr#2
asc-ΔC9	ascr#3
Glc-asc-C6-MK	ascr#4
asc-ωC3	ascr#5
asc-ΔC7-PABA	ascr#8
asc-C5	asc#9
IC-asc-C5	icas#9
asc-C9	ascr#10
asc-C4	ascr#11
asc-C6	ascr#12
asc-C11	ascr#18
asc-C11-EA	easc#18

Figure 1. Generalized life cycle of *Steinernema* nematodes



RESEARCH OBJECTIVES

The overarching goal of this study was to determine the role of *Xenorhabdus* symbionts on the development and on the ascaroside production of their *Steinernema* hosts. Specific objectives were as follows:

Objective 1: To investigate the role of *Xenorhabdus* symbionts on the time to maturation and sex ratio of first-generation *Steinernema* adults

Hypothesis: *Steinernema* nematodes associated with non-cognate *Xenorhabdus* symbionts, or non-symbiotic *Serratia*, will take longer to develop than cognate pairings.

Objective 2: To investigate the effect of *Xenorhabdus* symbionts on gonad development of first-generation *Steinernema* males and females.

Hypothesis: *Steinernema* nematodes associated with non-cognate *Xenorhabdus* symbionts, or non-symbiotic *Serratia*, will have smaller or underdeveloped gonads.

Objective 3: To investigate the role of *Xenorhabdus* symbionts on ascaroside production of first-generation *Steinernema* nematodes.

Hypothesis: *Steinernema* nematodes associated with non-cognate *Xenorhabdus* symbionts, or non-symbiotic *Serratia*, will have a different ascaroside profile (both qualitatively and quantitatively) when compared with the cognate *Xenorhabdus* pairing.

CHAPTER 2: INFLUENCE OF *XENORHABDUS* SYMBIONTS ON GONAD DEVELOPMENT IN *STEINERNEMA* NEMATODES

Abstract

Steinernema nematodes and their *Xenorhabdus* partners form an obligate mutualistic association. This partnership is insecticidal to a wide range of insects. *Steinernema* rely on their *Xenorhabdus* partner to produce toxins inside the insect cadaver to liberate nutrients from the insect, as well as antimicrobials to sterilize the cadaver, thus creating a suitable environment for reproduction. In return, *Steinernema* vector their *Xenorhabdus* between insect hosts. Disruption of this partnership may affect the success of both partners. For instance, when *Steinernema* associates with non-cognate symbionts, their virulence and reproductive fitness are affected. In this study, we examined the effect of symbiotic (cognate and non-cognate) and non-symbiotic bacteria on maturation time, gonad development, and sex ratio of first-generation *Steinernema* adults. Two *Steinernema* spp. were considered: *S. feltiae* SN and *S. carpocapsae* All. *In vitro* assays were carried out by pairing each nematode sp. with symbiotic (cognate and non-cognate) *Xenorhabdus*, and with non-symbiotic bacteria (*Serratia proteamaculans*). Additionally, for comparative purposes, we also considered adult nematodes reared *in vivo* in *Galleria mellonella* larvae to assess nematode development under natural conditions. Results from this study showed non-symbiotic *Serratia proteamaculans* did not support adult development of *S. feltiae* but it allowed development of *S. carpocapsae* adults. Sex ratio decreased from 2:1 to 1:1 (female: male) when *S. carpocapsae* adults were reared with the non-symbiotic *S. proteamaculans.* Cognate or non-cognate *Xenorhabdus* spp. and/or strains did not change the sex ratio of any of either *Steinernema* spp. tested. Morphometric analysis also revealed that bacterial conditions influenced adult size and gonad development in both *Steinernema* species. Body size (length

and width), and gonad length in both *S. feltiae* males and females, were significantly reduced when reared with a non-cognate *Xenorhabdus* species. In *S. carpocapsae*, males exhibited an enhanced body size (length and width) and gonad length when reared with a non-cognate *X. nematophila* strain. *S. carpocapsae* females also exhibited an enhanced gonad length when reared with a non-cognate *X. nematophila* strain. *S. carpocapsae* males and females were underdeveloped when reared with the non-symbiotic *S. proteamaculans*, and exhibited reduced body sizes and gonad lengths. We conclude that development of first-generation adults of both *Steinernema* spp. tested, in particular time to adult maturation as well as body and gonad size were directly influenced by the bacterial symbionts they were cultured with. However, response to the culture conditions was species specific.

1. Introduction

Steinernema nematodes have an obligate mutualistic association with *Xenorhabdus* bacteria (Boemare et al., 1993; Gaugler and Kaya, 1993; Boemare 2002). Third-stage infective juveniles, IJs, vector *Xenorhabdus* inside a specialized receptacle in the anterior portion of the intestine known as the "bacterial receptacle" (Bird and Akhurst, 1983; Snyder et al., 2007). Once a suitable insect host is found, IJs enter the insect via natural orifices and migrate to the hemocoel. Once there, IJs transition to a recovery phase, they exsheath and open their mouth and anus. Presence of the IJs in the insect's hemolymph triggers peristaltic movement of their intestine, contributing to the release of the bacteria via their anus (Snyder et al., 2007). *Xenorhabdus* produce an arsenal of toxins and secondary metabolites that cause a massive septicemia, killing the insect host in 24-48 hours. The bacteria and disintegrated insect tissues provide nutrients for the nematodes to mature and multiply (Akhurst 1983, Boemare et al. 1993).

Xenorhabdus also produce secondary metabolites with antimicrobial properties that not only kill the insect's natural microflora, but also prevent the cadaver from attack by saprobic microbes (i.e. bacteria and fungi), and predators that can potentially interfere with the completion of the nematodes life cycle (Akhurst, 1982; Chen et al., 1994; Boemare, 2002). *Xenorhabdus* also become the nematodes' sole food source (Boemare et al., 2002), allowing them to mature to first-generation adults and copulate. Fertilized females lay eggs in the cadaver, from which hatch first-stage juveniles. Approximately 2-3 generations occur inside the insect cadaver depending on host size and food availability. First-generation adults are the largest, with subsequent adult generations being morphologically similar, but smaller in size (Nguyen et al. 2007).

Chemical cues, such as the accumulation of carbon dioxide and ammonia inside the insect cadaver, are thought to lead to IJ formation and emergence from the insect cadaver (San-Blas et al., 2008).

Symbiont-host relationships are oftentimes tight, and disruption of the relationship has repercussions to the interacting partners (McMullen et al., 2017). Furthermore, in other mutualistic partnerships, studies have also shown that the symbiotic partners play a key role in normal development of their hosts. For example, Montgomery and McFall-Ngai (1994) demonstrated that *Vibrio fischeri*, the bacterial symbiont of the bobtail squid, *Euprymna scolopes*, induces morphogenesis of the light organ in juvenile squids. Furthermore, the absence of the symbiont leads to arrested postembryonic development of the light organ (Montgomery and McFall-Ngai, 1994).

In another early study, Costa et al. (1993) noticed that treatment of female B-biotype sweetpotato whiteflies, *Bemisia tabaci*, with the antibiotic oxytetreacycline hydrochloride led to adverse effects. Specifically, the authors noted that offspring development of the treated females was adversely affected when the insects were depleted of their bacterial symbionts. Additionally, the mycetomes, symbiont-housing structures, were reduced in size. Overall, the offspring of the antibiotic-treated females exhibited a reduced capacity to cause disease in plants (Costa et al., 1993). Furthermore, Costa et al. (1997) revealed that in *Bemisia aregntifolii,* antibiotics that interfere specifically with bacterial protein synthesis had an adverse effect on offspring development and reduced the number of offspring that became adult whiteflies.

In filarial nematodes, a few studies have shown that absence of *Wolbachia* symbionts, which reside in the ovaries and uterus of females (Kozek, 1977; Kozek, 2005), affects reproduction and progeny development (Hoerauf et al., 1999). For example, Hoerauf et al. (1999)

demonstrated that the removal of *Wolbachia* with the antibiotic tetracycline leads to infertility and the inhibition of embryogenesis in *Litomosoides sigmodontis*. In relation to this, Landmann et al. (2011) demonstrated that the immediate response to the removal of *Wolbachia* in *Brugia malayi,* another filarial worm, was apoptosis in the adult germline, somatic cells of the embryos and microfilariae. The authors concluded that the pattern of apoptosis correlates with processes that are affected by *Wolbachia* depletion, e.g. perturbed embryogenesis and sterilization (Landmann et al., 2011).

With respect to entomopathogenic nematodes, a few studies have demonstrated a reduction in progeny production when the nematodes associated with non-cognate *Xenorhabdus* partners (Sicard et al., 2004; Murfin et al., 2015; McMullen et al., 2017). For example, Sicard et al. (2004) investigated the level of specificity between *S. carpocapsae* and *X. nematophila* by pairing the nematodes with non-cognate *Xenorhabdus* and *Photorhabdus* spp. Their study revealed nematode progeny was impacted when they associated with non-cognate bacteria. Additionally, the rearing of the nematodes with a non-native symbiont affected symbiont transmission (Sicard et al., 2004).

Murfin et al. (2015) also demonstrated that the reproductive fitness (i.e. progeny number, productive infection number) of *Steinernema feltiae* nematodes was enhanced when they associated with their cognate symbiont or with strains that are closely related to their cognate symbiont. Similarly, McMullen et al. (2017) showed that association with non-cognate *Xenorhabdus* symbionts impact the virulence and progeny production in different *Steinernema* spp. that are hosts of *X. bovienii* symbionts. Specifically, the authors showed that association with non-cognate symbionts had a significant negative impact on survival and virulence of IJs and progeny production (McMullen et al., 2017).

Altogether these studies demonstrated that *Steinernema* fitness is greater when the nematodes associated with their cognate symbionts, or with symbionts that are more closely related to their native partners (Sicard et al., 2004; Murfin et al., 2015; McMullen et al., 2017).

In an early study by Poinar and Thomas (1966), the authors anecdotally reported that reproductive organs of *S. carpocapsae* "appeared degenerate" when reared in the absence *Xenorhabdus*, thus suggesting that bacterial symbionts may play a role in gonad development. However, until now, it is yet unknown if *Xenorhabdus,* as the nematodes food source, have an impact on adult development. In this respect, we herein investigated the effect of symbiotic (cognate and non-cognate), and non-symbiotic bacteria have on gonad development, maturation time, and sex ratio of first-generation adults of two *Steinernema* spp.: *S. carpocapsae* All strain and *S. feltiae* SN strain.

2. Materials and Methods

2.1 Rearing of nematode cultures

Two nematode species were considered in this study: *S. carpocapsae* All (Sierra Biologics) strain and *S. feltiae* SN strain. Nematodes were reared *in vivo* utilizing the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) according to procedures described by Kaya and Stock (1997). Briefly, a nematode suspension of 1.8 mL (approximately 1,000-2,000 IJs/mL was pipetted onto a 10cm Petri dish lined with two discs of filter paper (Whatman #1). Approximately 10-12 *G. mellonella* larvae were added to the plate, which was covered and stored at 25 ºC in the dark. Upon death, cadavers were placed in a modified White trap according to procedures outlined by Kaya and Stock (1997). Once infective juveniles (IJs) emerged into the White trap, they were collected, rinsed with sterile distilled water, and stored in

tissue culture flasks at a concentration of 1,000-2,000 IJs/mL. Flasks were stored at 15 ºC ± 1ºC in the dark until needed.

2.2 Rearing of bacteria

Four *Xenorhabdus* species: *X. nematophila* (symbiont of *S. carpocapsae* All strain [XnAll]), *X. nematophila* (symbiont of *S. anatoliense* [XnAna]), *X. bovienii* (symbiont of *S. feltiae* SN strain [XbfSN]), *X. bovienii* (symbiont of *S. puntauvense* [Xbp]) were considered in the experiments. Additionally, one non-symbiotic bacterium, *Serratia proteamaculans* was used in these experiments (Table 1).

Bacterial cultures were grown up from glycerol stocks in 15 mL centrifuge tubes containing 5 mL of Luria Bertani (LB) broth supplemented with 0.1% (w/v) overnight for 20-24 hours with agitation at 28ºC ± 1 ºC. Overnight bacterial cultures were diluted with LB broth to an average optical density of approximately 0.8. An aliquot 50 µL of the standardized bacterial culture was pipetted onto the 3.5 cm lipid agar plates supplemented with 0.1% sodium pyruvate (w/v), and spread into a lawn. The culture was streaked onto nutrient agar supplemented with 0.0025% (w/v) bromothymol blue, 0.004% (w/v) triphenyltetrazolium, and 0.1% (w/v) sodium pyruvate (NBTA) to confirm phenotypic identity, and verify the catalase profile (catalase-negative). Three plates per bacterial species were made. Plates were stored upside down at 28ºC ± 1 ºC for 2 days.

2.3 Preliminary in vivo *assays*

In vivo assays were carried out to estimate time to maturation and sex ratio of first-generation adults of the two *Steinernema* spp. considered in this study. For this purpose, a 6-

well plate was considered as the experimental arena following procedures described by Kaya and Stock (1997). Briefly, a one single filter paper disc (Whatman #1) was placed into each well. A 100 µL inoculum containing 200-250 IJs was dispensed into each well. One *G. mellonella* larva weighing between 15-0.25 g was placed in each well. The well-plates were closed with the lid and stored at room temperature (23ºC ± 1 ºC). After 2.5-3 days, *G. mellonella* cadavers were removed from the plates and dissected in M9 buffer. Adults were collect for morphometric analysis (see supplemental documents). Sex ratio was determined by sieving the adults through a 20 mesh, to remove insect debris, and performing volumetric counts. This experiment was repeated three times.

2.4 Collection of Steinernema *aposymbiotic eggs*

Nematodes were reared *in vivo* as described in section 2.1. For *S. feltiae*, cadavers were dissected 3 days post-infection, whereas for *S. carpocapsae,* cadavers were dissected 3.5-4 days post-infection to collect gravid females. Dissection time was based on results from assays described in section 2.3. Approximately 300 females were collected with the assistance of an L-shape needle and placed into a watch glass containing 10-15 mL of M9 buffer. Females were rinsed and subsequently ground in an axenizing NaOCl solution that facilitated dissolution of the females' bodies allowing egg release (McMullen and Stock, 2014). Egg pellets were thoroughly rinsed with sterile distilled water and concentrated by centrifugation at 1600 rpm for 3 minutes (McMullen and Stock, 2014).

2.5 Experimental setup

An inoculum of 100 µL of *Steinernema* egg suspension was pipetted onto either Petri dishes with the tested bacteria, or Petri dishes containing solely lipid agar as the negative control. There were 2 plates per nematode-bacterium combination per experimental setup. The egg aliquot contained an average of 1,000 to 2,000 eggs/plate for *S. feltiae* and *S. carpocapsae,* respectively. Dishes were stored at 25ºC ± 1 ºC for 18-24 hours to allow nematode development from eggs. Plates were then removed from the incubator and maintained at room temperature (23ºC ± 1 ºC) to allow the juveniles development. The experiment was repeated at least three times (Fig. 1).

2.6 In vitro *harvesting of first-generation of adults*

The timeframe for collection of adults varied between 3-7 days, depending on the bacterial treatment the nematodes were reared in. Adults were collected from the dishes by rinsing the adults off the dishes with M9 solution into a Petri dish. Adults from each plate and treatment were collected in M9 buffer and placed into a 15 mL centrifuge tubes (BD Falcon®) to determine the total volume of the adult/M9 mixture. The mixture was vortexed so adults were homogenous throughout the mixture, and volumetric counts were performed, noting the number of females and males to determine the sex ratio. The total number of adults was determined from the counts utilizing the equation $C_1V_1=C_2V_2$.

2.7 Morphometric analysis

In each experimental replicate, a total of 30 adults (15 males and 15 non-gravid females) per bacterial treatment were collected using an L-shaped needle for morphometric analysis (Kaya

and Stock, 1997). Nematodes were fixed in triethanolamine:formalin fixative (TAF) (Courtney et al., 1955) according to procedures described by Stock and Goodrich-Blair (2012). Males and females were individually mounted onto slides and visualized under an Olympus BX51 microscope equipped with DIC optics and a digital imaged for morphometric analysis with MicroSuite FIVE software (Center Valley, PA).

For the females, the following parameters were measured: TBL (total body length), MBW (maximum body width), AE-G (distance from anterior end to anterior portion of gonad), AGL (anterior gonad length), and PGL (posterior gonad length) (Fig. 2), TGL (total gonad length= sum of anterior and posterior gonad length), and G (total gonad length/total body length). For males, the following morphometric parameters were measured: TBL (total body length), MBW (maximum body width), AE-G (distance from anterior end to anterior portion of the testis flexure), TGL (total gonad length), TREF (testis reflexion length) (Fig. 3), and G (total gonad length/total body length). For immature stages collected off plates when adults did not develop, only TBL (total body length) and MBW (maximum body width) were measured.

2.8 Statistical analysis

Statistical analysis of morphometric data was done with JMP software, version 13 (SAS). A mixed-effects ANOVA model (α=0.05) was considered for the morphometric analysis. In the mixed-effects model, experimental setup was defined as a random effect and bacterial condition was defined as a fixed effect to account for any possible variability in setup. This was carried out through the fit model function, utilizing a restricted maximum likelihood (REML) method. A *post hoc* least squares means Tukey's honest significant difference (HSD) analysis was conducted to determine bacterial effects. Females and males for each species were analyzed

separately. For both males and females TBL (total body length), MBW (maximum body width), TGL (total gonad length= sum of anterior and posterior gonad length), and G (total gonad length/total body length) were the parameters of interest. For males, an additional parameter TREF (testis reflexion length, an area of mitotic and meiotic spermatocyte division) was analyzed.

3. Results

3.1 Time to adult development

For either *Steinernema* spp. considered, the average time to adulthood was 3 days when they were reared *in vitro* with their cognate symbiont. This timeframe was comparable to the maturation time observed in *in vivo* reared nematodes (Table 2). However, rearing of *S. carpocapsae* with non-cognate *X. bovienii* feltiae SN strain delayed adult maturation to 4 days (Table 2). Non-symbiotic *S. proteamaculans* scantily supported development of *S. carpocapsae*. Adult maturation was delayed to 7 days when the nematodes were reared with non-symbiotic *S. proteamaculans*. Furthermore, adult numbers were significantly low when compared with those reared with *Xenorhabdus* bacteria (cognate and non-cognate).

Adult development of *S. feltiae* was also delayed when the nematodes were reared with a non-cognate species, *X. nematophila* All strain. *S. proteamaculans* did not support adult development in this *Steinernema* species. None of the tested species were able to mature in the absence of bacteria (Table 2).

3.2 Sex ratio

Sex ratio of both *S. carpocapsae* and *S. feltiae* first-generation adults when reared *in vitro* with their cognate symbionts was female biased, with an average of 1,264 females and 574 males, and 492 females and 315 males, respectively (Table 2). Sex ratio of *in vitro* reared *S. carpocapsae* remained unaltered and female biased at 2:1 (female: male) when the nematodes were cultured with either cognate or non-cognate *Xenorhabdus* (Table 2). Adult numbers of *S. carpocapsae* were reduced 10-fold when cultured with non-symbiotic *S. proteamaculans*. Under this condition, the female: male ratio was 1:1 (Table 2). For all *in vitro* rearing conditions, except for the non-symbiotic bacteria condition, adult yield was higher than that observed *in vivo*. *S. carpocapsae* nematodes reared *in vivo* exhibited a 1.5:1 (female: male) ratio.

Sex ratio of *S. feltiae* remained unaltered and female biased at 1.5:1 (female: male) when the nematodes were cultured *in vitro* with either cognate or non-cognate *Xenorhabdus* (Table 2). For all *in vitro* rearing conditions, adult yield was higher than that observed *in vivo*. Interestingly, adult numbers were highest when the nematodes were cultured *in vitro* with a non-cognate symbiotic strain, *X. bovienii* puntauvense strain (Xbp). *S. feltiae* nematodes reared *in vivo* exhibited a 1:1 (female: male) ratio.

3.3 Steinernema *morphometrics under different* in vitro *conditions*

Body width, gonad length, and the G value of *S. carpocapsae* females were significantly affected when the nematodes were reared *in vitro* with cognate and non-cognate *Xenorhabdus* symbionts. In particular, when the nematodes were exposed to a non-cognate symbiotic species, XbfSN, MBW was reduced (Fig. 4; Fig. 5B). TGL was augmented when *S. carpocapsae* females were reared with a non-cognate symbiotic strain, XnAna (Fig. 5C). The value of G was

augmented when *S. carpocapsae* females were reared with either non-cognate *Xenorhabdus* (Fig. 5D). TBL was not significantly different when *S. carpocapsae* females were reared on either non-cognate symbiont (Fig. 5A). Females were underdeveloped under non-symbiotic bacteria exhibiting a reduced body size (TBL and MBW). Similarly, TGL and the G value, were also reduced in comparison to any of the other *in vitro* bacterial conditions (Table 3, Fig. 4, Fig. 5).

With respect to *S. carpocapsae* males, all morphometric parameters measured were significantly impacted when the nematodes were cultured *in vitro* and with non-cognate *Xenorhabdus* symbionts (Table 3; Fig. 6; Fig. 7). Interestingly, TBL increased when males were exposed to either of the non-cognate symbionts (Fig. 7A). Additionally, an increase in MBW, TREF, and TGL was observed when males were exposed to the non-cognate symbiotic strain, XnAna (Fig. 7B, C, D). The G value was not significantly different when *S. carpocapsae* males were reared on either non-cognate symbiont (Fig. 7E). Males were significantly smaller (TBL and MBW), and exhibited a reduced TGL, TREF, and a lower G value when reared with non-symbiotic *S. proteamaculans* (Table 3; Fig. 6; Fig. 7).

Non-cognate *Xenorhabdus* symbionts had an effect on the size (TBL and width MBW) and TGL of *S. feltiae* females (Fig. 9). Specifically, TBL, MBW and TGL were reduced when females were cultured on the non-cognate symbiotic species XnAll (Table 4; Fig. 8; Fig. 9A, B, C). However, females reared with their cognate symbiont did not exhibit significant differences in the G value when compared to either of the non-cognate *Xenorhabdus* bacteria (Fig. 9D). Culturing of females on the non-cognate symbiotic strain Xbp significantly impacted MBW (Fig. 9). Females were wider when reared with the non-cognate symbiotic strain Xbp (Fig. 9B).

Most of the morphometric traits in *S. feltiae* males were affected when reared *in vitro* on the non-cognate symbiotic species XnAll (Fig. 11). Specifically, males were smaller (both TBL,

MBW), and exhibited a reduced TGL (Table 4, Fig. 10, Fig. 11A, B, D). Culturing of males on the non-cognate symbiotic strain Xbp did not significantly affect any of the morphometric values measured (Fig. 11A, B, C, D). The TREF and G value remained unaltered for any of the cognate or non-cognate rearing conditions (Fig. 11 D, E).

4. Discussion

The role of endosymbionts in the development and reproduction of their hosts has been widely studied in many groups of organisms. For example, in the bobtail squid, *Eurypema scolopes*, the absence of its *Vibrio fischeri* symbiont leads to abnormal postembryonic development of their light organs (Montgomery and McFall- Ngai, 1994). Additionally, in sweet potato whiteflies, the removal of their endosymbionts via antibiotic treatment has an effect on offspring development and reduces the number of adult whiteflies (Costa et al., 1997). With particular reference to Nematoda, it is now well known that *Wolbachia* endosymbionts drastically affects the embryogenesis and fertility of Filarid females (Hoerauf et al., 1997).

Furthermore, bacteria as a food source also impact the development of bacterivore nematodes, such as *Caenorhabditis elegans*. For example, the presence of bacteria inhibits dauer formation in this nematode, and at the same time promotes adult development (Golden and Riddle, 1982; Golden and Riddle, 1984). Additionally, a study by Montalvo-Katz et al. (2013) demonstrated that *C. elegans* fed on the bacterium *Bacillus megaterium*, they exhibited enhanced resistance to infection, and adult development was promoted. However, egg laying was impaired, suggesting that the bacterial food source may result in differences in development (Montalvo-Katz et al., 2013).

With respect to EPNs, Poinar and Thomas (1966) anecdotally reported that the gonads of *S. carpocapsae* (strain DD-136) “appeared degenerate” when reared without bacteria in *in insecta*, thus suggesting symbionts might play a role in gonad development. The authors further indicated that the nematodes did not reproduce when reared with non-symbiotic bacteria, such as *Bacillus cereus* and *Serratia marcescen*s, or without bacteria (Poinar and Thomas, 1966). In this respect, in this study we assessed the role of symbiotic (cognate and non-cognate) and non-symbiotic bacteria on the development of adults of first-generation adults of two *Steinernema* spp.: *S. carpocapsae* and *S. feltiae.*

Our results showed that in both nematode spp. tested, adults reared with cognate symbiotic bacteria, or with a non-cognate symbiotic strain, developed faster. Development time in *S. carpocapsae* reared with non-symbiotic *S. proteamaculans,* was delayed substantially to 7 days. Additionally, this bacterium did not sustain adult development in *S. feltiae*. Similarly, Ehlers et al (1990) noted that *S. carpocapsae* could develop on *Escherichia coli*, and that their development was also delayed, with only about half of the plates yielding adult stages.

Sex ratio of *in vitro* reared *S. carpocapsae* and *S. feltiae* remained unaltered and was female biased when the nematodes were cultured with either cognate or non-cognate *Xenorhabdus*. In this respect, Alsaiyah et al. (2009), who examined sex ratio of five different *Steinernema* species, including *S. carpocapsae* and *S. feltiae*, observed a similar female bias when reared or *in vitro*. The authors concluded that the female bias in the nematodes’ population facilitates male mating competition (Alsaiyah et al., 2009).

Results from this study showed that unlike *S. feltiae* juveniles, *S. carpocapsae* were unable to mature when reared with the non-symbiotic *S. proteamaculans, S. carpocapsae* was

able to mature. However, adult numbers were reduced and the female: male ratio decreased to 1:1.

Interestingly, the female: male sex ratio was altered when either *Steinernema* spp. was reared *in vivo*. *S. carpocapsae* nematodes reared *in vivo* exhibited a 1.5:1 (female: male) ratio compared to the 2:1 (female: male) ratio observed when *S. carpocapsae* was reared *in vitro* with *Xenorhabdus*. *S. feltiae* nematodes reared *in vivo* exhibited a balanced 1:1 (female: male) ratio compared to the 1.5:1 (female: male) ratio observed when *S. feltiae* was reared *in vitro* with *Xenorhabdus*. Contrastingly, Campos-Herrera and Gutiérrez (2014) reported that sex ratio in Spanish populations of *S. feltiae* was female-biased under *in vivo* culturing conditions. However, their observations were done after 7 days post-infection, which correlates with the presence of second-generation adults. Therefore, these observations cannot be correlated with the results from this study.

Morphometric analyses revealed that females of both *S. carpocapsae* and *S. feltiae* had smaller body widths (MBW) when reared with a non-cognate symbiotic species. *S. feltiae* females also exhibited a significantly smaller TBL when reared with a non-cognate symbiotic species, while *S. carpocapsae* did not. Rearing of *S. feltiae* females on non-cognate symbiotic species negatively affected TGL. Additionally, the G value in *S. feltiae* females was not significantly impacted when the nematodes were cultured on non-cognate *Xenorhabdus* spp and strains.

Males of *S. feltiae* exhibited a significant decrease in the values of TBL, MBW, and TGL when reared with the non-cognate symbiotic species (XnAll). The non-cognate symbiotic Xbp strain had no significant impact on any of these morphometric traits. Similar to females, the G value in *S. feltiae* males was not significantly impacted when the nematodes were cultured on

non-cognate *Xenorhabdus* spp. and strains. Additionally, TREF length was unaltered when *S. feltiae* males were reared on any of the *Xenorhabdus* symbionts.

Contrastingly, *S. carpocapsae* females showed an augmented TGL when they grew with non-cognate symbiotic XnAna strain. Additionally, the value G was augmented when *S. carpocapsae* females were reared on either non-cognate *Xenorhabdus.* The augmented G value suggests that the females' gonads occupied the majority of their body cavity, thus leading to an increase in gonad length, which may benefit their egg production. Overall, males exhibited an increase in their body size (TBL) when exposed to either to the non-cognate *Xenorhabdus* strains and species. Additionally, the values of MBW, TREF and the TGL were higher when males were exposed to the non-cognate symbiotic XnAna strain.

As mentioned before, *S. proteamaculans* sustained growth of *S. carpocapsae*; however, both males and females were smaller (TBL and MBW). Additionally, when both males and females were reared with this non-symbiotic bacterium, a reduction in all morphometric traits measured was observed.

In conclusion, results from this study confirmed that *Xenorhabdus* symbionts as a food source play an important role in the maturation time and development of first generation adults of *Steinernema* nematodes. However, their impact varies depending on the *Steinernema-Xenorhabdus* pairs considered. In this study, *S. feltiae* demonstrated a tighter level of dependence for its native symbiont, while *S. carpocapsae* was more promiscuous and able to mature with either non-cognate or non-symbiotic bacteria. The observed symbiont specificity in *S. feltiae* has been corroborated in other *S. feltiae* fitness studies (Murfin et al., 2015). Additionally, the looser association between *S. carpocapsae* and its symbiont has been observed in previous *S. carpocapsae* studies (Ehlers et al., 1990; Sicard et al., 2004). However, these

results are specific, and other *Steinernema-Xenorhabdus* pairings should be considered. Our observations also confirmed that rearing conditions are critical for nematode development and maturation time. Specifically, first generation adults of both of the studied species were always smaller than those cultured *in vivo* (Table S1-S2, Fig.S1-S8). In this respect, Miranda et al. (2013) demonstrated that insect host diet has a significant effect on the production of IJ progeny in the insect cadavers. Thus, the insect host in this tripartite symbiosis is a key player in the life cycle of *Steinernema* nematodes, and cannot be disregarded. Future research should focus on elucidating the specific nutritional contributions of the insect host to sustain growth, development and overall fitness of *Steinernema* nematodes.

Table 1. *Steinernema*-Bacteria Pairings Considered

***Steinernema* species and strain**	**Bacterial species**	**Status**
Steinernema carpocapsae All strain	*Xenorhabdus nematophila* symbiont associated with *S. carpocapsae* All strain (XnAll)	Cognate symbiont
	Xenorhabdus nematophila symbiont associated with *S. anatoliense* (XnAna)	Non-cognate symbiotic strain
	Xenorhabdus bovienii associated with *S. feltiae* SN strain (XbfSN)	Non-cognate symbiotic species
	Serratia proteamaculans	Non-symbiotic bacteria
Steinernema feltiae SN strain	*Xenorhabdus bovienii* associated with *S. feltiae* SN strain (XbfSN)	Cognate symbiont
	Xenorhabdus bovienii associated with *S. puntauvense* Li6 strain (Xbp)	Non-cognate symbiotic strain
	Xenorhabdus nematophila symbiont associated with *S. carpocapsae* All strain (XnAll)	Non-cognate symbiotic species
	Serratia proteamaculans	Non-symbiotic bacteria

Table 2. Time to adult maturation and sex ratio of *S. carpocapsae* and *S. feltiae* under different culture conditions

Nematode species	Rearing condition	Maturation time	Number of females*	Number of males*	Sex ratio (female: male)
Steinernema carpocapsae	*in vivo*	3 days	195-343 (282 ± 77)	171-267 (204 ± 54)	1.5:1
	in vitro with XnAll	3 days	574-2250 (1,264 ± 744)	278-1050 (574 ± 333)	2:1
	in vitro with XnAna	3 days	619-1,910 (1,370 ± 671)	214-1,280 (768 ± 534)	2:1
	in vitro with XbfSN	4 days	179-3,227 (1,210 ± 1,375)	220-1,800 (664 ± 761)	2:1
	in vitro with *S. proteamaculans*	7 days	2-55 (25 ± 27)	2-50 (22 ± 25)	1:1
Steinernema feltiae	*in vivo*	3 days	180-280 (219 ± 54)	183-350 (254 ± 86)	1:1
	in vitro with XbfSN	3 days	163-775 (492 ± 302)	170-440 (315 ± 138)	1.5:1
	in vitro with Xbp	3 days	310-3,600 (1,524 ± 1,536)	300-1,975 (942 ± 783)	1.5:1
	in vitro with XnAll	4 days	130-450 (259 ± 136)	100-375 (183 ± 129)	1.5:1
	in vitro with *S. proteamaculans*	N/A	N/A	N/A	N/A**

*Depicts minimum-maximum (average ± standard deviation) to encompass the data spread
**Only one underdeveloped adult was anecdotally detected

Table 3. Morphometric traits of *S. carpocapsae* adults reared *in vitro* with symbiotic (cognate and non-cognate) and non-symbiotic bacteria

Morphometric traits	XnAll		XnAna		XbfSN		*S. proteamaculans*	
	Females (n=45)	Males (n=45)	Females (n=45)	Males (n=45	Females (n=45)	Males (n=45)	Females (n=27)	Males (n=20)
TBL	1,423-2,945 (2,021 ± 424.6)	881-1,290 (1,066 ± 95.3)	1,568-2,743 (2,069 ± 320.2)	963-1,479 (1,200 ± 126.5)	1,412-2,567 (1,904 ± 270.7)	913-1,456 (1,163 ± 108.8)	541-1523 (985 ± 319.4)	485-851 (684 ± 110.8)
MBW	104-249 (145 ± 34.5)	55-97 (75 ± 9.3)	112-230 (144 ± 25.5)	61-96 (81 ± 8.3)	91-184 (131 ± 24.5)	57-95 (75 ± 9.3)	36-101 (67 ± 22.3)	39-60 (49 ± 6.1)
TREF	--	142-284 (203 ± 29.5)	--	128-323 (227 ± 47.3)	--	104-274 (201 ± 34.5)	--	65-171 (119 ± 30.6)
TGL	1,639-4,436 (2,750 ± 715.1)	647-1,021 (844 ± 82.1)	1,748-4,933 (3,033 ± 820.7)	735-1,242 (974 ± 132.8)	1,728-5,037 (2,835 ± 649.9)	677-1,271 (901 ± 111.9)	39-1640 (704 ± 486.3)	282-693 (459 ± 113.1)
G	1.0-1.7 (1.4 ± 0.2)	0.71-0.91 (0.79 ± 0.044)	1.1-1.9 (1.4 ± 0.2)	0.72-0.93 (0.81 ± 0.047)	1.1-2.2 (1.5 ± 0.2)	0.68-0.87 (0.77 ± 0.049)	0.1-1.3 (0.6 ± 0.3)	0.53-0.96 (0.67 ± 0.092)

All measurements are in μM. All data shown presents the measurements according to this format: minimum-maximum (average ± standard deviation) to encompass the spread of the data.

Table 4. Morphometric traits of *S. feltiae* adults reared *in vitro* with symbiotic (cognate and non-cognate) bacteria

Morphometric traits	XbfSN		Xbp		XnAll	
	Females (n=45)	Males (n=45)	Females (n=45)	Males (n=45)	Females (n=45)	Males (n=45)
TBL	1,360-2,893 (2,085 ± 306.3)	858-1,283 (1,070 ± 106.3)	1,495-2,957 (2,168 ± 327.2)	939-1,291 (1,076 ± 83.1)	1,107-2,248 (1,736 ± 250.6)	764-1,266 (945 ± 107.2)
MBW	87-139 (115 ± 11.3)	60-87 (75 ± 6.5)	92-210 (123±18.7)	60-82 (72 ± 6.3)	82-126 (99±10.4)	49-86 (65 ± 7.5)
TREF	--	116-342 (205 ± 43.8)	--	132-257 (193 ± 30.9)	--	135-242 (188 ± 24.4)
TGL	1,653-4,067 (2,863 ± 565.4)	557-1,296 (907 ± 126.9)	1,624-4,334 (2,919 ± 639.6)	669-1,102 (880 ± 102.0)	1,441-3,940 (2,439 ± 480.5)	583-1,101 (782 ± 102.7)
G	1.0-1.6 (1.4 ± 0.1)	0.64-1.01 (0.85 ± 0.070)	0.9-1.5 (1.3 ± 0.1)	0.66-0.92 (0.82 ± 0.057)	1.1-1.8 (1.4 ± 0.2)	0.72-0.98 (0.83 ± 0.058)

All measurements are in μM. All data shown presents the measurements according to this format: minimum-maximum (average ± standard deviation) to encompass the spread of the data.

Figure 1. Schematic representation of experimental design

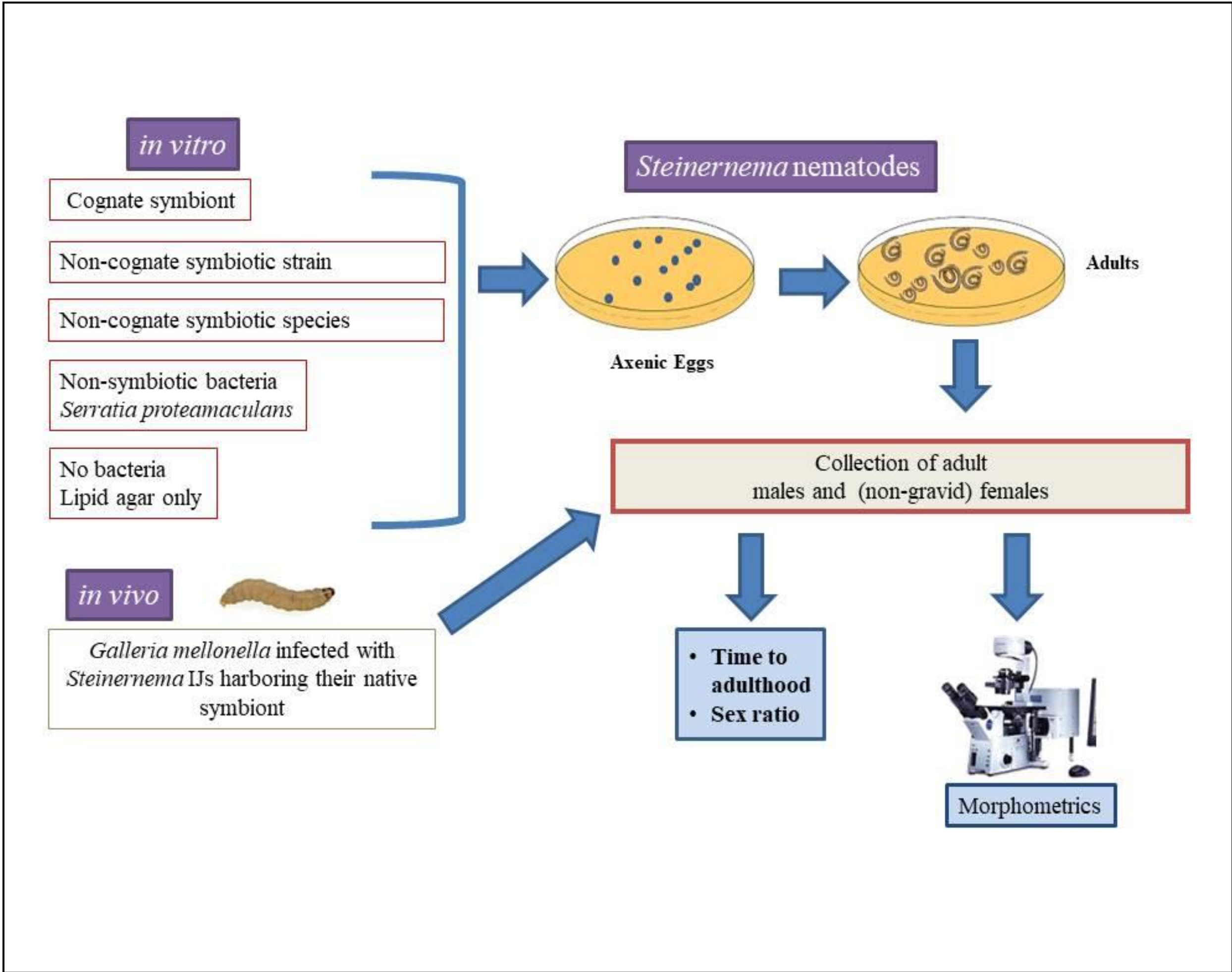


Figure 2. Schematic representation of morphometric traits measured in first-generation *Steinernema* females

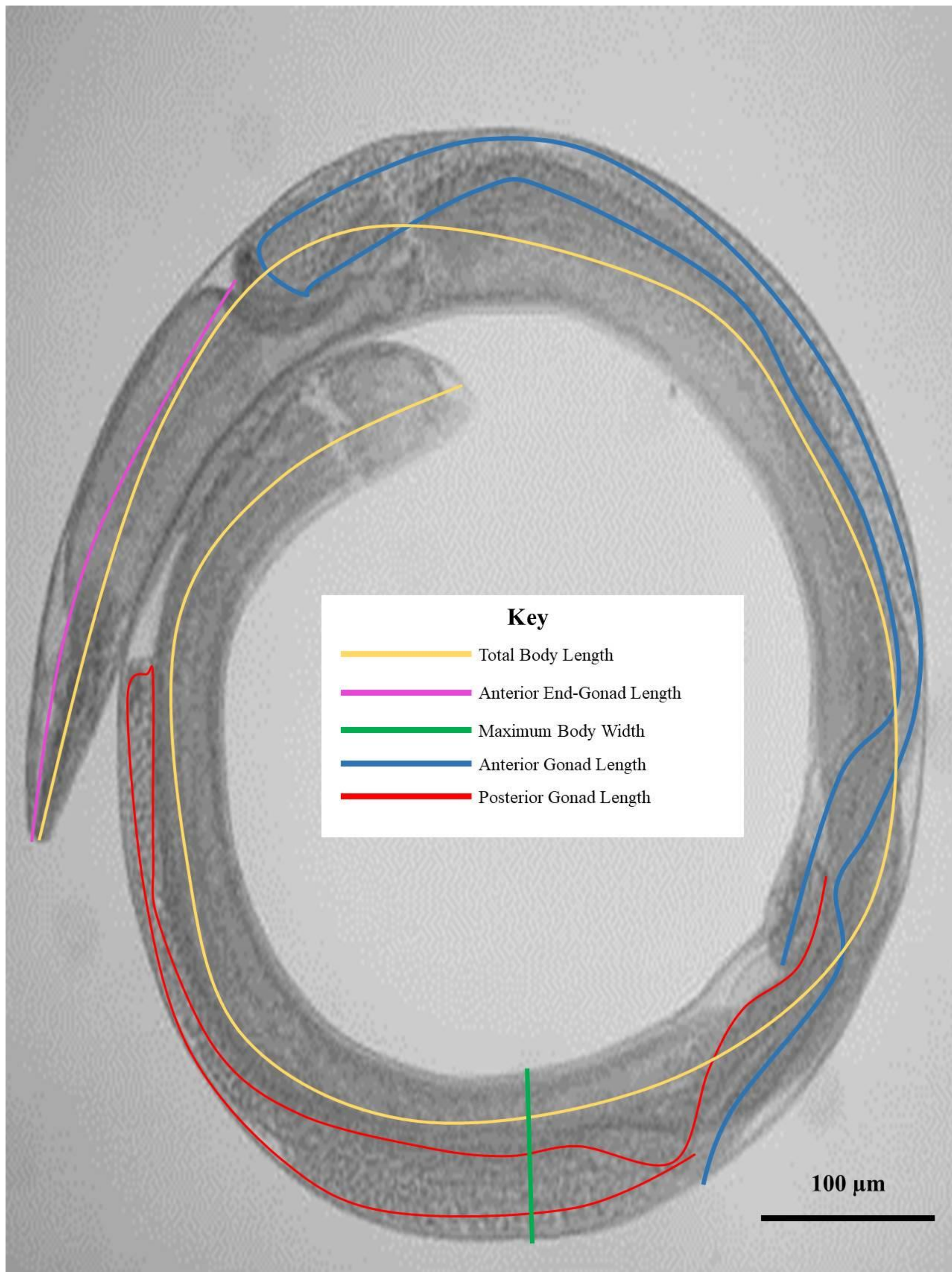


Figure 3. Schematic representation of morphometric traits measured in first-generation *Steinernema* males

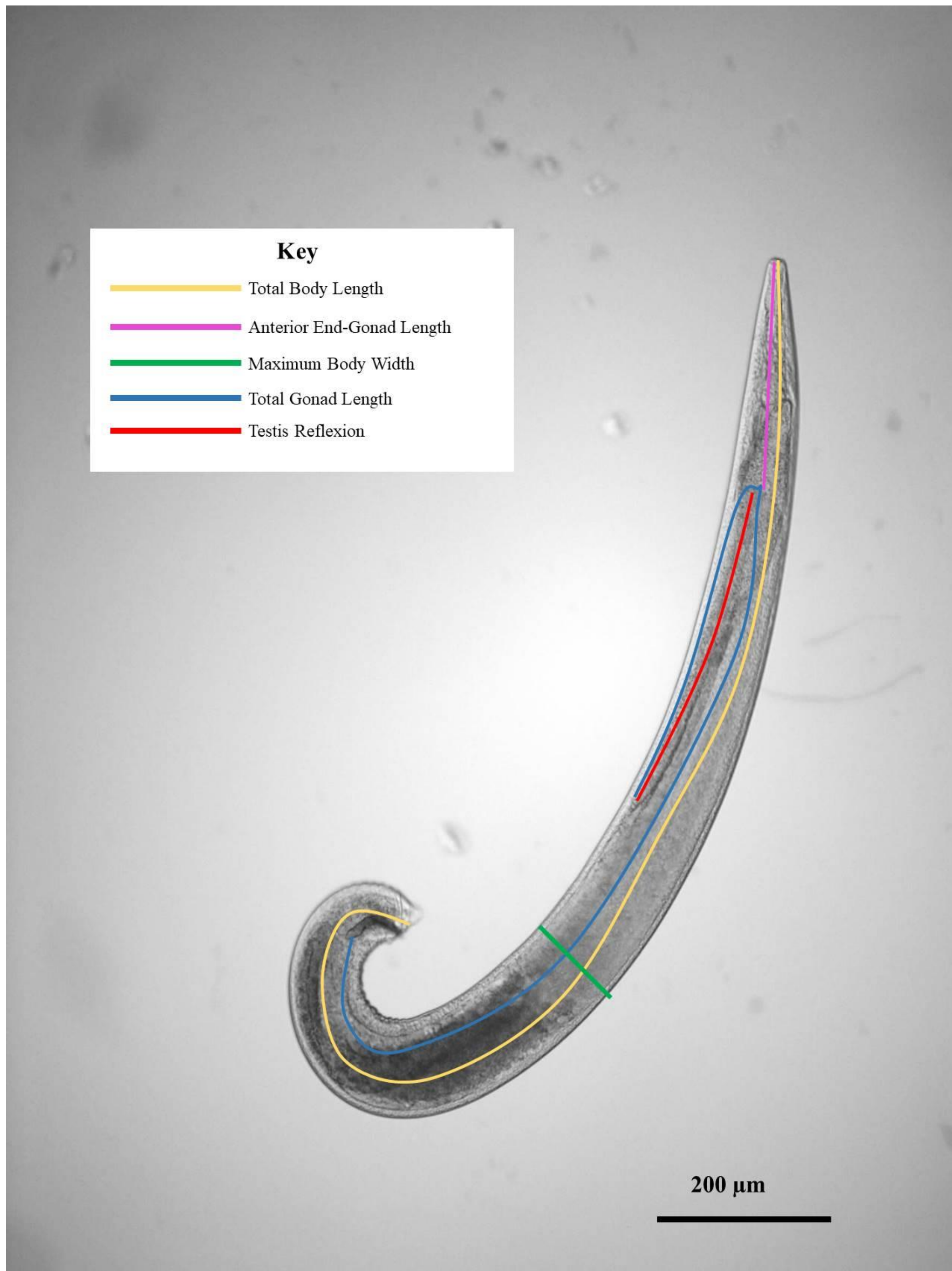
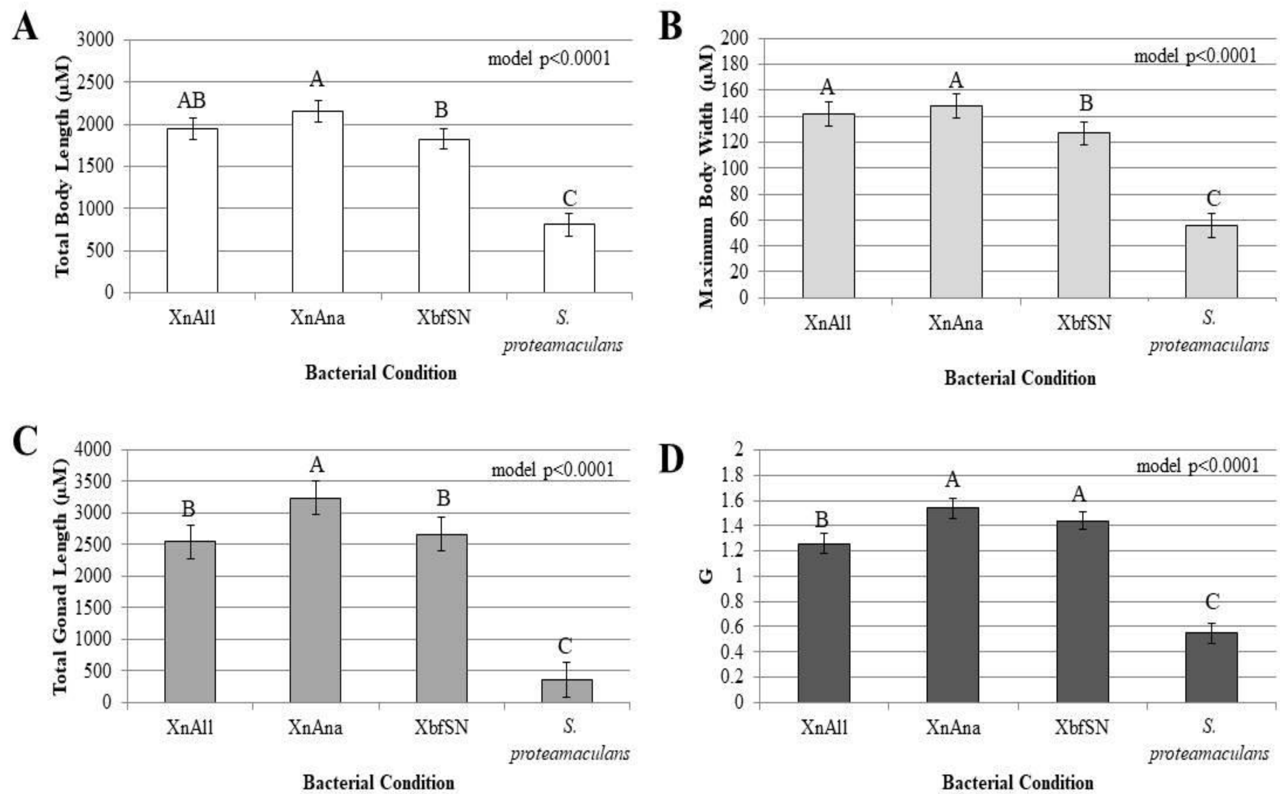


Figure 4. *S. carpocapsae* females reared *in vitro* under different culture conditions

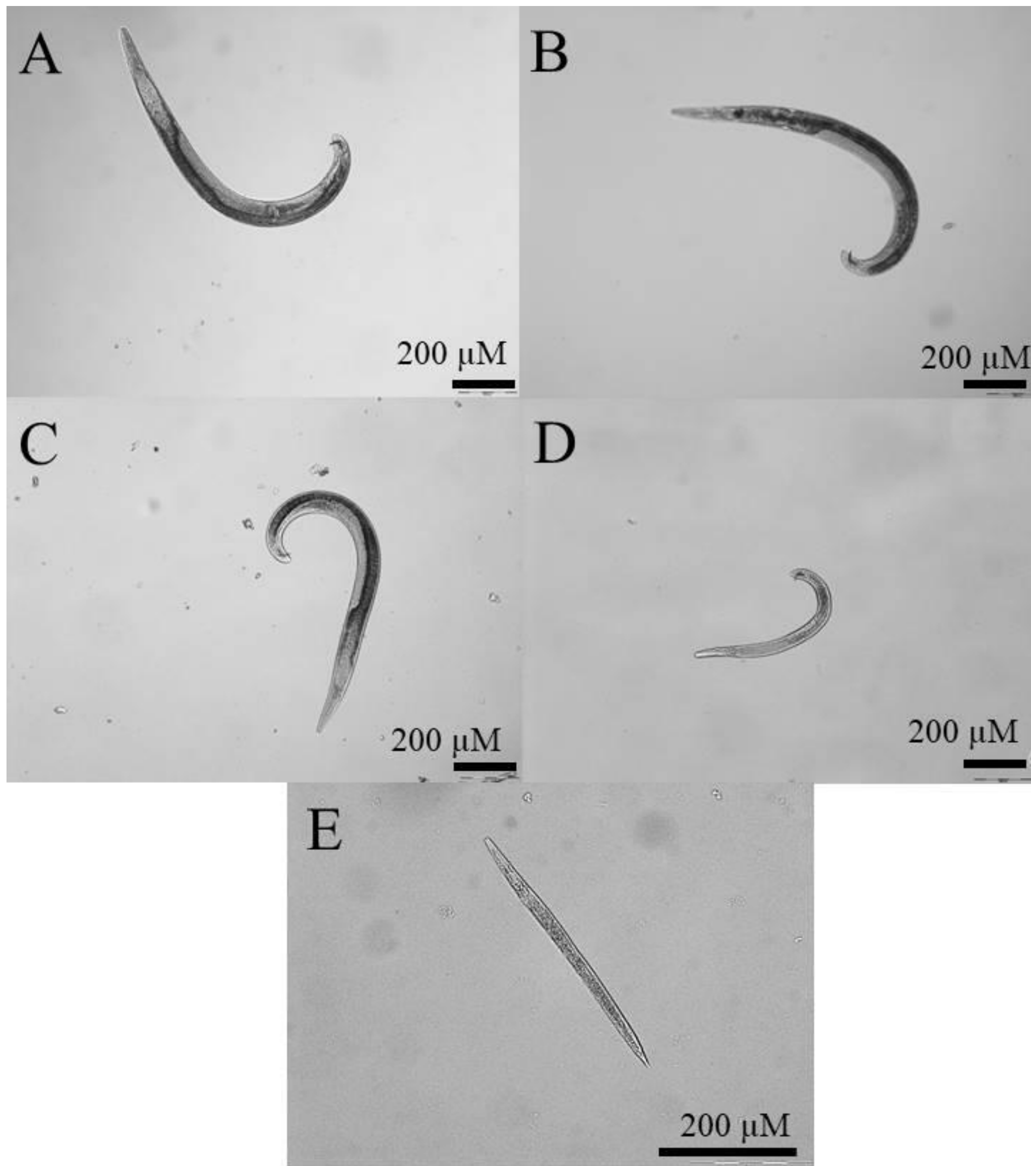
A. reared with cognate XnAll. **B.** reared with non-cognate strain, XnAna. **C.** reared with non-cognate species, XbfSN. **D.** reared with non-symbiotic *Serratia proteamaculans*. **E.** reared with no bacteria (juvenile stage).

Figure 5. Comparison of morphometric parameters of *S. carpocapsae* females reared with symbiotic (cognate and non-cognate) and non-symbiotic bacteria



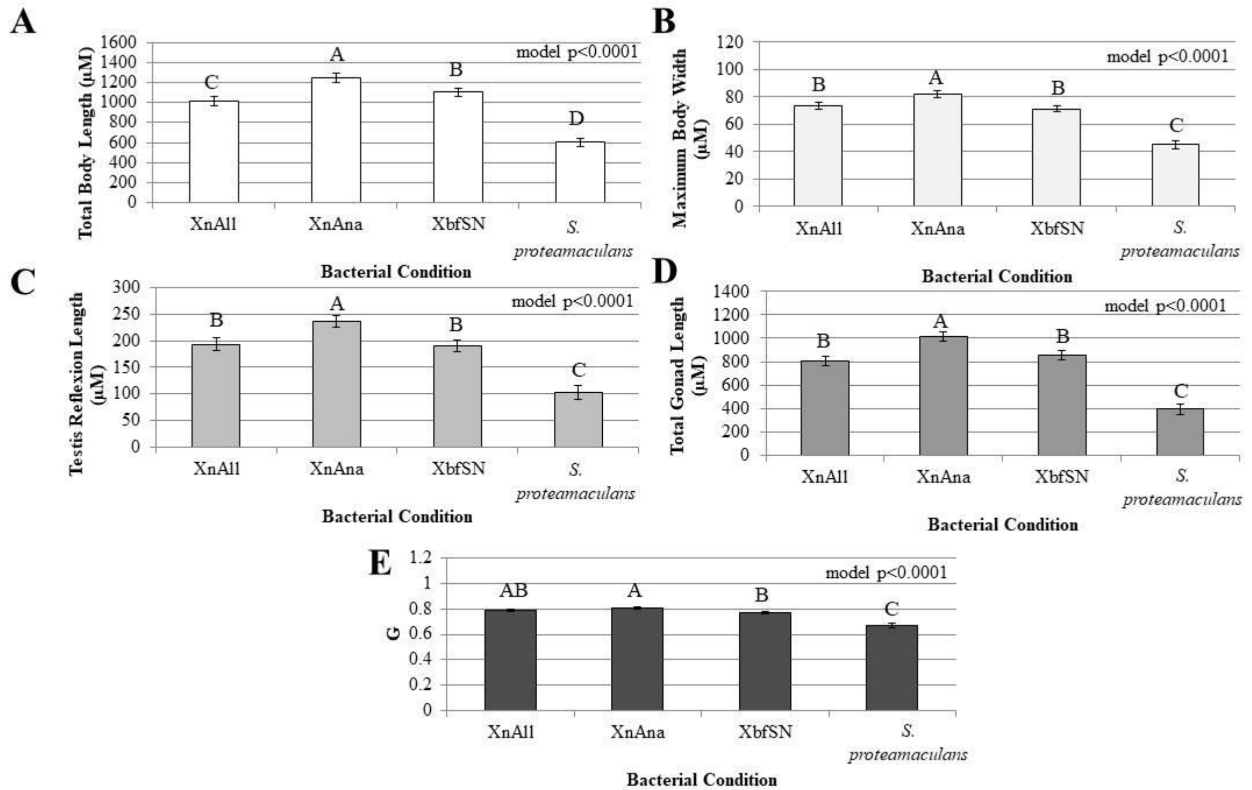
A. Total body length, **B.** Maximum body width, **C.** Total gonad length, and **D.** G (gonad length/body length). A mixed-effects ANOVA model with α=0.05 was run treating experimental setup as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Tukey's HSD analysis was performed.

Figure 6. *S. carpocapsae* males reared *in vitro* under different culture conditions



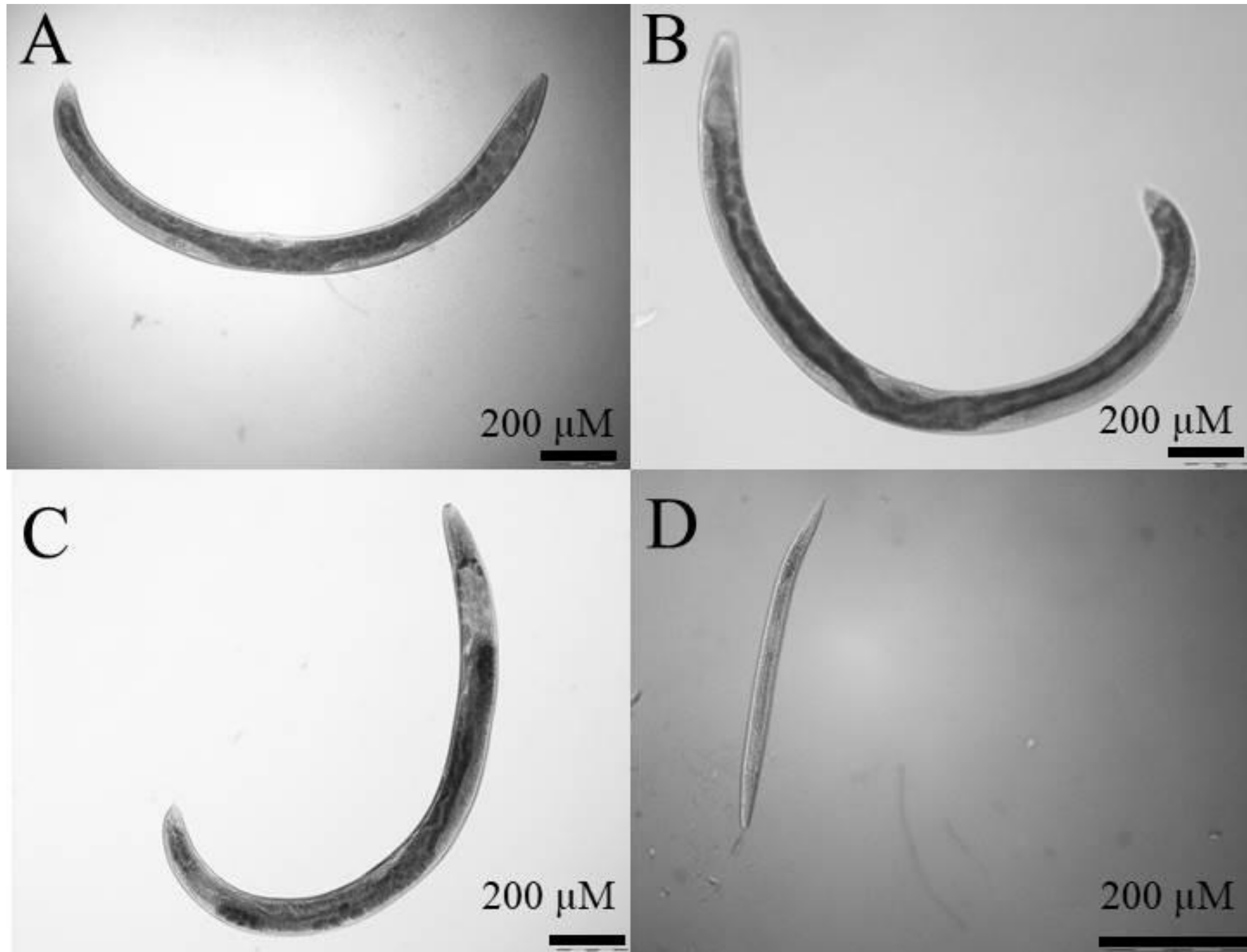
A. reared with cognate XnAll. **B.** reared with non-cognate strain, XnAna. **C.** reared with non-cognate species, XbfSN. **D.** reared with non-symbiotic *Serratia proteamaculans*. **E.** reared with no bacteria (juvenile stage).

Figure 7. Comparison of morphometric parameters of *S. carpocapsae* males reared with symbiotic (cognate and non-cognate) and non-symbiotic bacteria



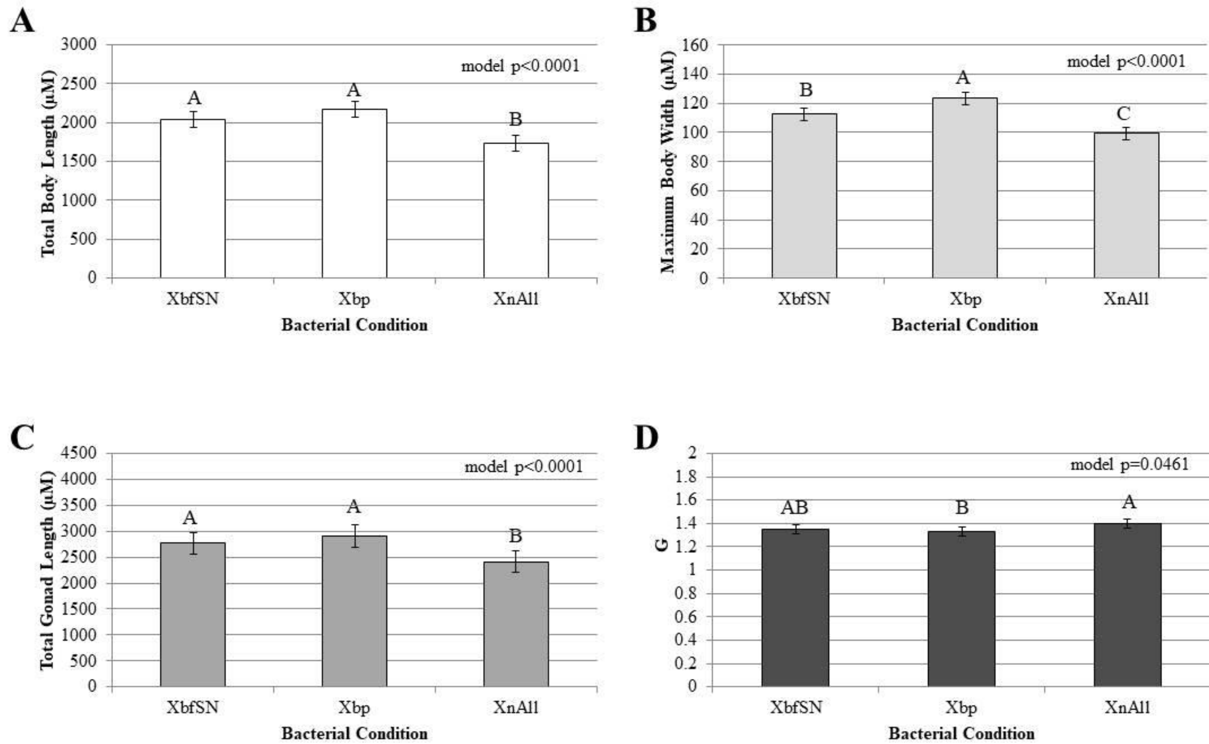
A. Total body length, **B.** Maximum body width, **C.** Total gonad length, and **D.** G (gonad length/body length). A mixed-effects ANOVA model with α=0.05 was run treating experimental setup as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Tukey's HSD analysis was performed.

Figure 8. *S. feltiae* females reared *in vitro* under different culture conditions



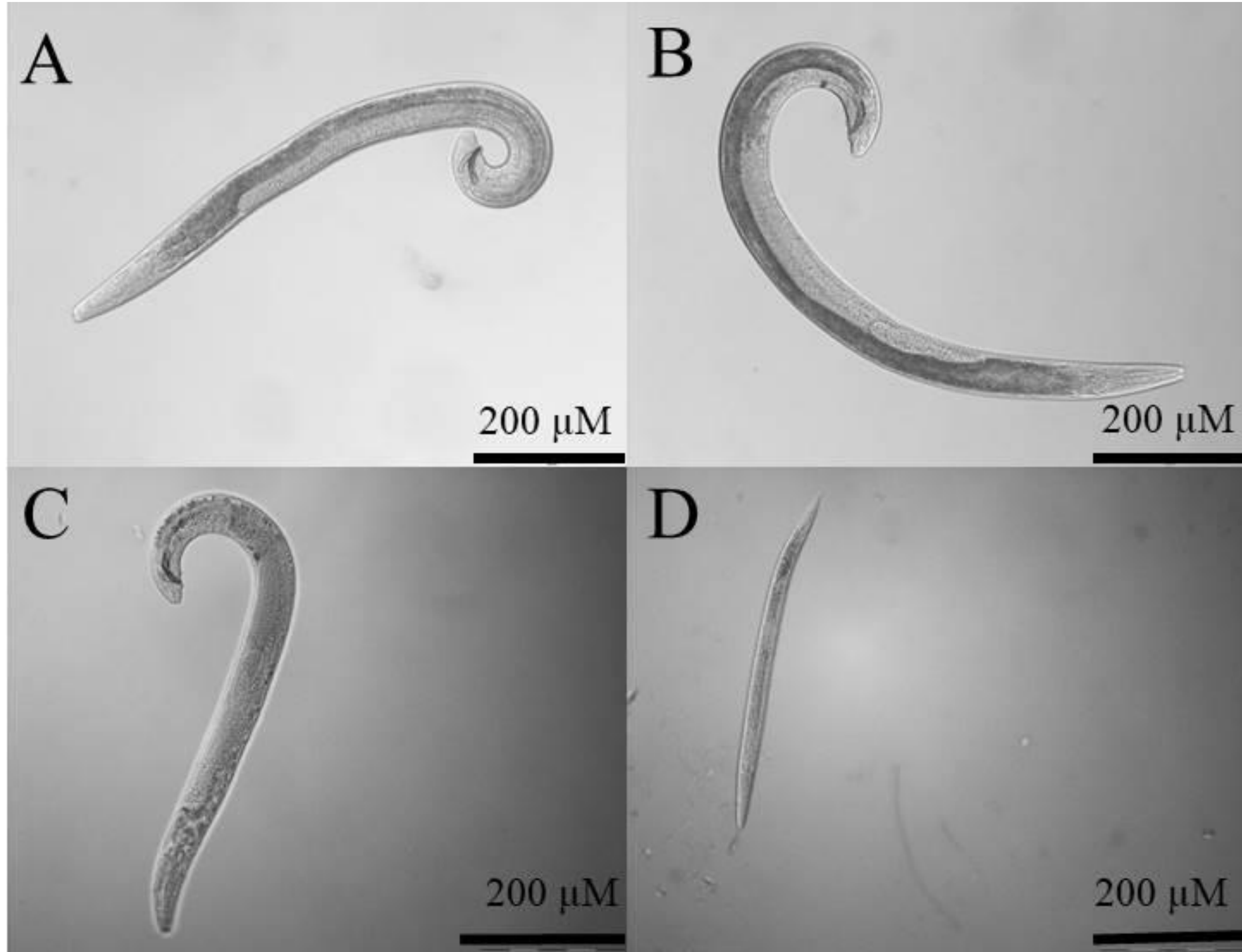
A. reared with cognate XbfSN. **B.** reared with non-cognate strain, Xbp. **C.** reared with non-cognate species, XnAll. **D.** reared with no bacteria (juvenile stage).

Figure 9. Comparison of morphometric parameters of *S. feltiae* females reared with cognate and non-cognate symbiotic bacteria



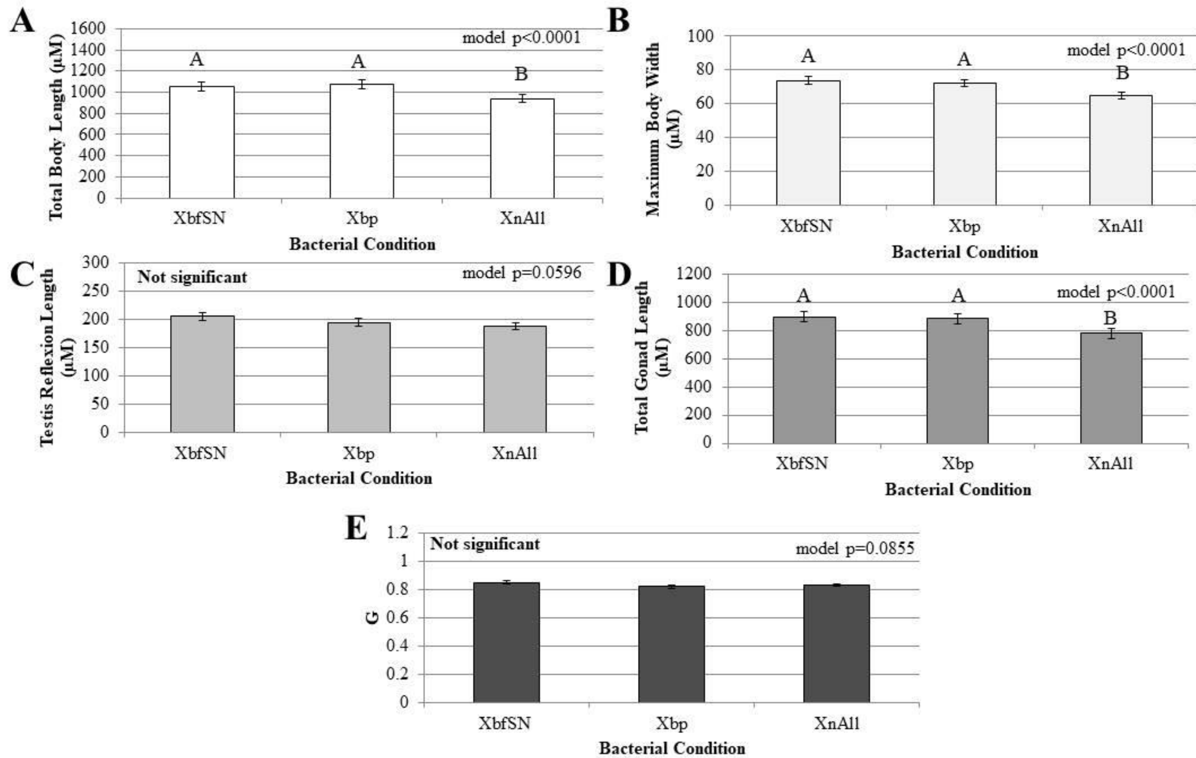
A. Total body length, **B.** Maximum body width, **C.** Total gonad length, and **D.** G (gonad length/body length). A mixed-effects ANOVA model with α=0.05 was run treating experimental setup as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Tukey's HSD analysis was performed. The *Serratia* condition did not support adult development.

Figure 10. *S. feltiae* males reared *in vitro* under different culture conditions



A. reared with cognate XbfSN. **B.** reared with non-cognate strain, Xbp. **C.** reared with non-cognate species, XnAll. **D.** reared with no bacteria (juvenile stage).

Figure 11. Comparison of morphometric parameters of *S. feltiae* males reared with cognate and non-cognate symbiotic bacteria



A. Total body length, **B.** Maximum body width, **C.** Testis reflexion, **D.** Total gonad length, and **E.** G (gonad length/body length). A mixed-effects ANOVA model with α=0.05 was run treating experimental setup as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Tukey's HSD analysis was performed. The *Serratia* condition did not support adult development.

Supplemental Documents

We measured *Steinernema* nematodes harboring their cognate symbiont reared in *Galleria mellonella* larvae to compare morphometric traits with *in vitro* reared nematodes with their cognate symbiont. *In vivo* assays were setup as described in section 2.3 and morphometric analysis was performed as described in section 2.7.

Morphometric data from *in vivo*-reared nematodes were used for comparative purposes. A mixed-effects model with the replicate defined as a random effect and bacterial condition defined as a fixed effect was utilized. A Student's T-test, with an $\alpha=0.05$ was utilized to compare *in vivo* reared nematodes to nematodes reared *in vitro* on their cognate symbiont.

Table S1. Comparison of morphometric traits measured of *in vitro* reared *S. carpocapsae* with their cognate symbiont and *in vivo* reared *S. carpocapsae*

Morphometric traits	**Female**		**Male**	
	in vitro with XnAll (n=45)	*in vivo* (n=45)	*in vitro* with XnAll (n=45)	*in vivo* (n=45)
TBL	1,423-2,945 (2,021 ± 424.6)	1,248-5,901 (2,807 ± 897.9)	881-1,290 (1,066 ± 95.3)	1,169-2,048 (1,587 ± 212.1)
MBW	104-249 (145 ± 34.5)	104-256 (158 ± 33.8)	55-97 (75 ± 9.3)	88-182 (134 ± 19.9)
TREF	N/A	N/A	142-284 (203 ± 29.5)	281-689 (434 ± 91.5)
TGL	1,639-4,436 (2,750 ± 715.1)	1,486-9,162 (4,232 ± 1,459.8)	647-1,021 (844 ± 82.1)	1,219-2,196 (1,653 ± 213.9)
G	1.0-1.7 (1.4 ± 0.2)	1.2-1.8 (1.5 ± 0.1)	0.71-0.91 (0.79 ± 0.044)	0.91-1.20 (1.04 ± 0.069)

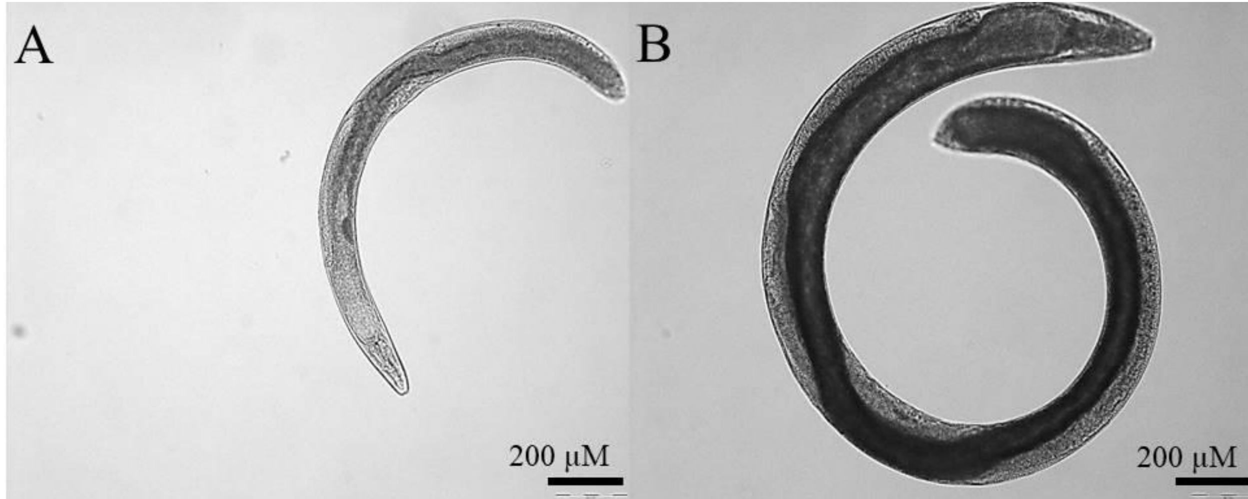
All morphometrics are in µM. All data shown presents the measurements according to this format: minimum-maximum (average ± standard deviation) to encompass the spread of the data.

Table S2. Comparison of morphometric traits measured of *in vitro* reared *S. feltiae* with their cognate symbiont and *in vivo* reared *S. feltiae*

Morphometric traits	**Female**		**Male**	
	in vitro with XbfSN (n=45)	*in vivo* (n=45)	*in vitro* with XbfSN (n=45)	*in vivo* (n=45)
TBL	1,360-2,893 (2,085 ± 306.3)	1,739-4,526 (2,668 ± 627.2)	858-1,283 (1,070 ± 106.3)	1,110-1,702 (1,364 ± 145.8)
MBW	87-139 (115 ± 11.3)	124-242 (166 ± 26.7)	60-87 (75 ± 6.5)	94-149 (115 ± 10.8)
TREF	N/A	N/A	116-342 (205 ± 43.8)	238-505 (365 ± 65.5)
TGL	1,653-4,067 (2,863 ± 565.4)	2,686-7,983 (4,383 ± 1,098.1)	556.82-1,296 (907 ± 126.9)	1,184-1,808 (1,424 ± 157.2)
G	1.0-1.6 (1.4±0.1)	1.3-1.9 (1.6±0.1)	0.64-1.01 (0.85 ± 0.070)	0.90-1.21 (1.05 ± 0.72)

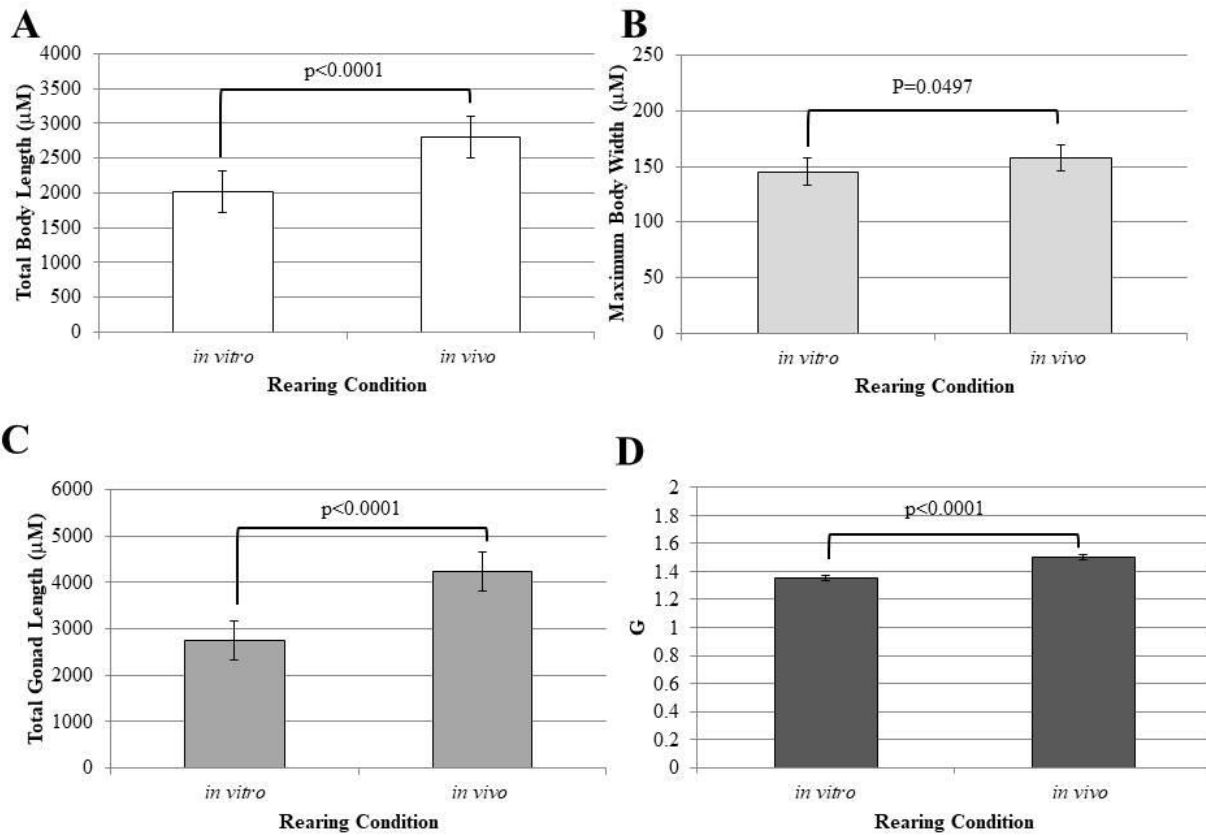
All morphometrics are in μM. All data shown presents the measurements according to this format: minimum-maximum (average ± standard deviation) to encompass the spread of the data.

Figure S1. *S. carpocapsae* females under different rearing conditions



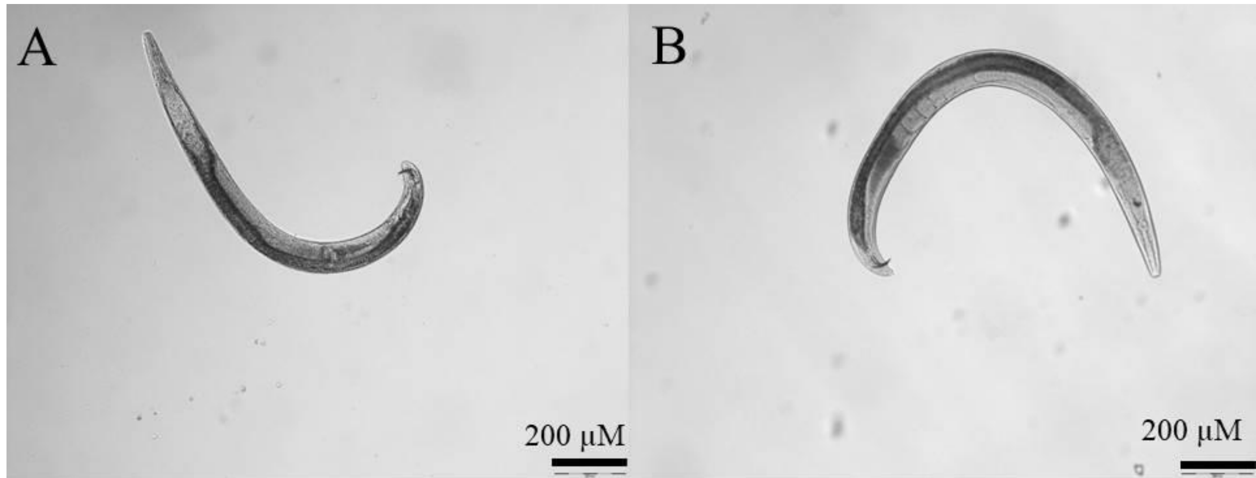
A. Female reared *in vitro* with cognate *X. nematophila*, **B.** Female colonized with cognate *X. nematophila* reared *in vivo*

Figure S2. Comparison of morphometric traits measured of *S. carpocapsae* females reared *in vitro* with their cognate symbiont and reared *in vivo*



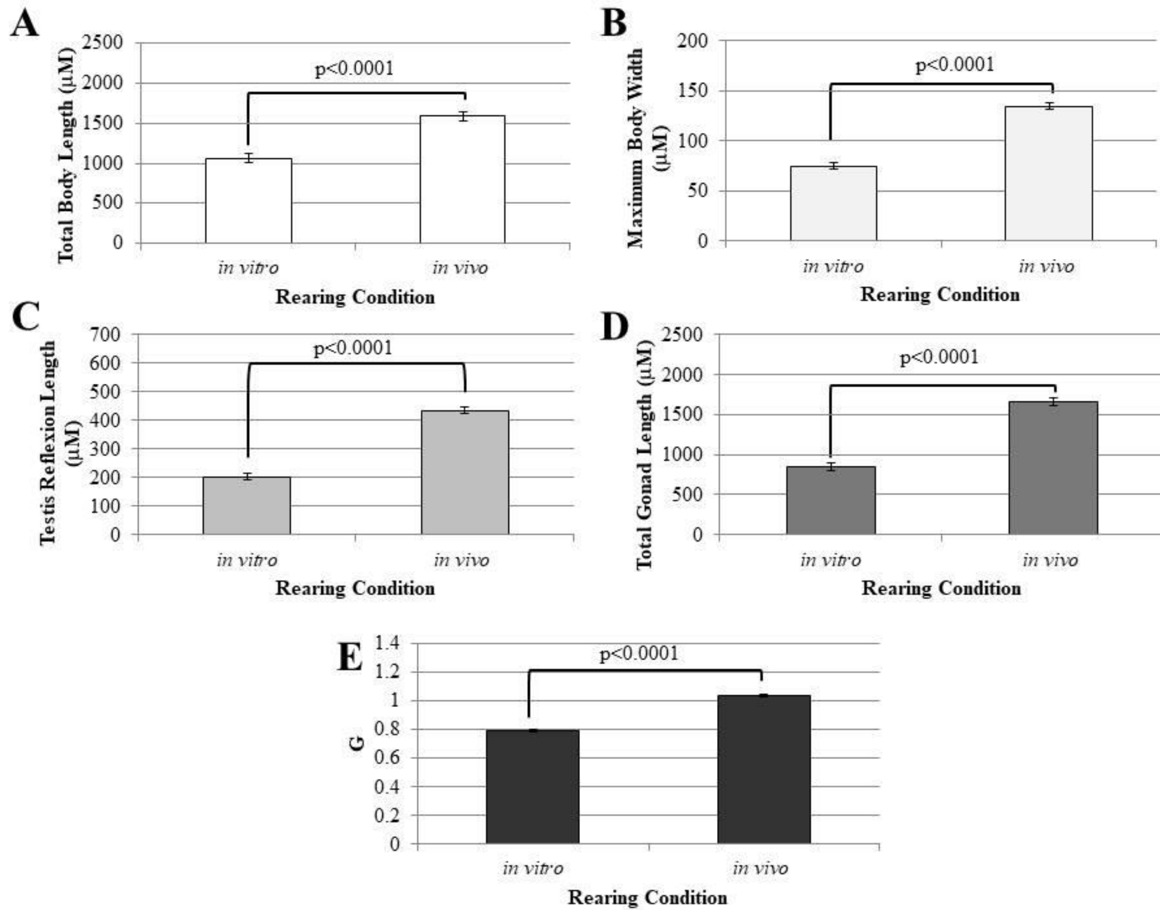
A. Total body length, **B.** Maximum body width, **C.** Total gonad length, and **D.** G (gonad length/body length). A mixed-effects model with α=0.05 was run treating replicate as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Student's t-test analysis was performed.

Figure S3. *S. carpocapsae* males under different rearing conditions



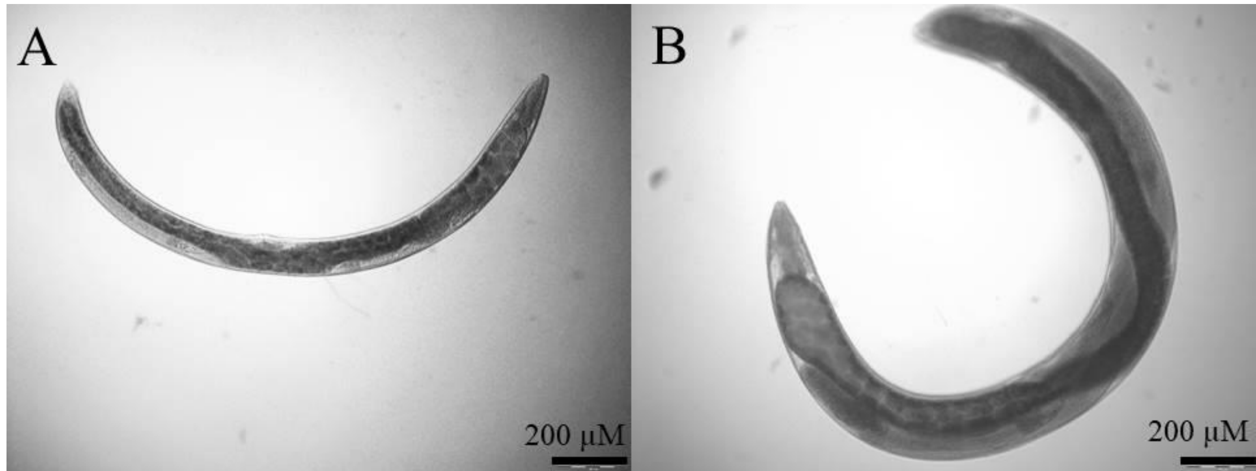
A. Male reared *in vitro* with cognate *X. nematophila*, **B.** Male colonized with cognate *X. nematophila* reared *in vivo*

Figure S4. Comparison of morphometric traits measured of *S. carpocapsae* males reared *in vitro* with their cognate symbiont and reared *in vivo*



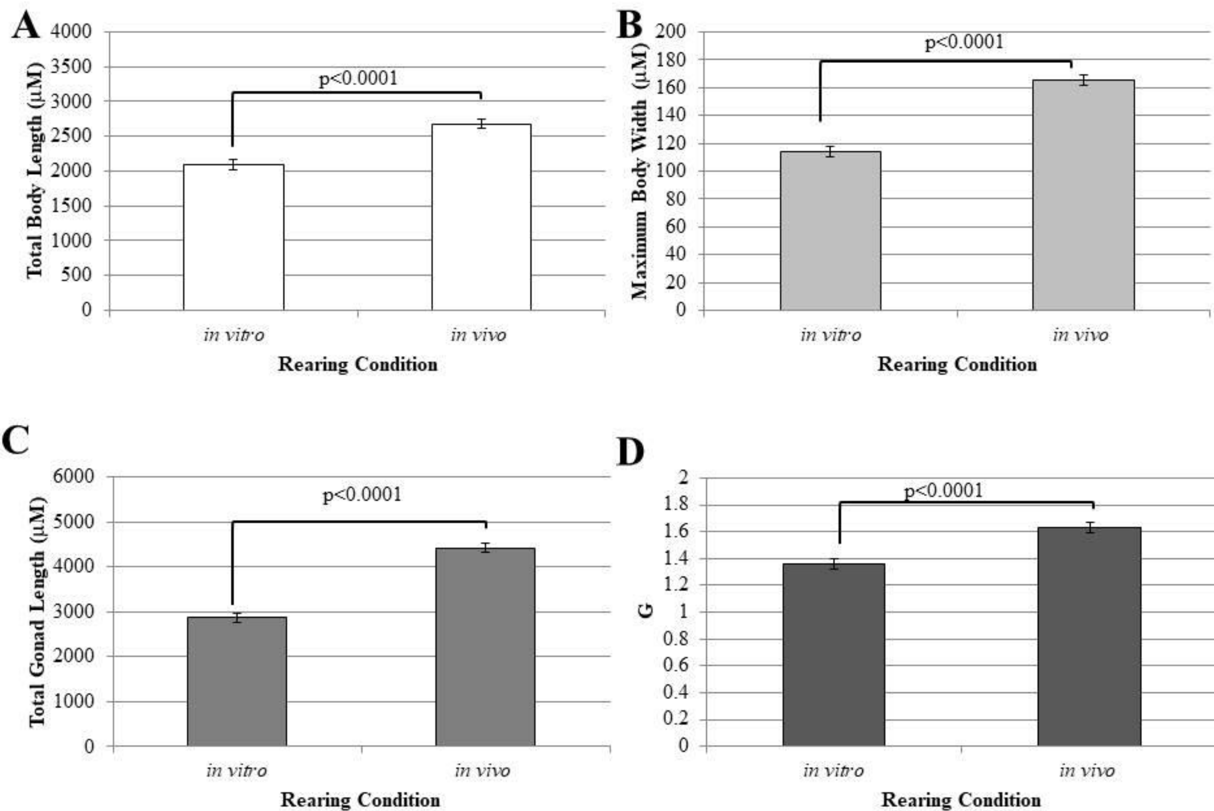
A. Total body length, **B.** Maximum body width, **C.** Testis reflexion, **D.** Total gonad length and **E.** G (gonad length/body length). A mixed-effects model with $\alpha=0.05$ was run treating replicate as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Student's t-test analysis was performed.

Figure S5. *S. feltiae* females under different rearing conditions



A. Female reared *in vitro* with cognate *X. bovienii*, **B.** Female colonized with cognate *X. bovienii* reared *in vivo*

Figure S6. Comparison of morphometric traits measured of *S. feltiae* females reared *in vitro* with their cognate symbiont and reared *in vivo*

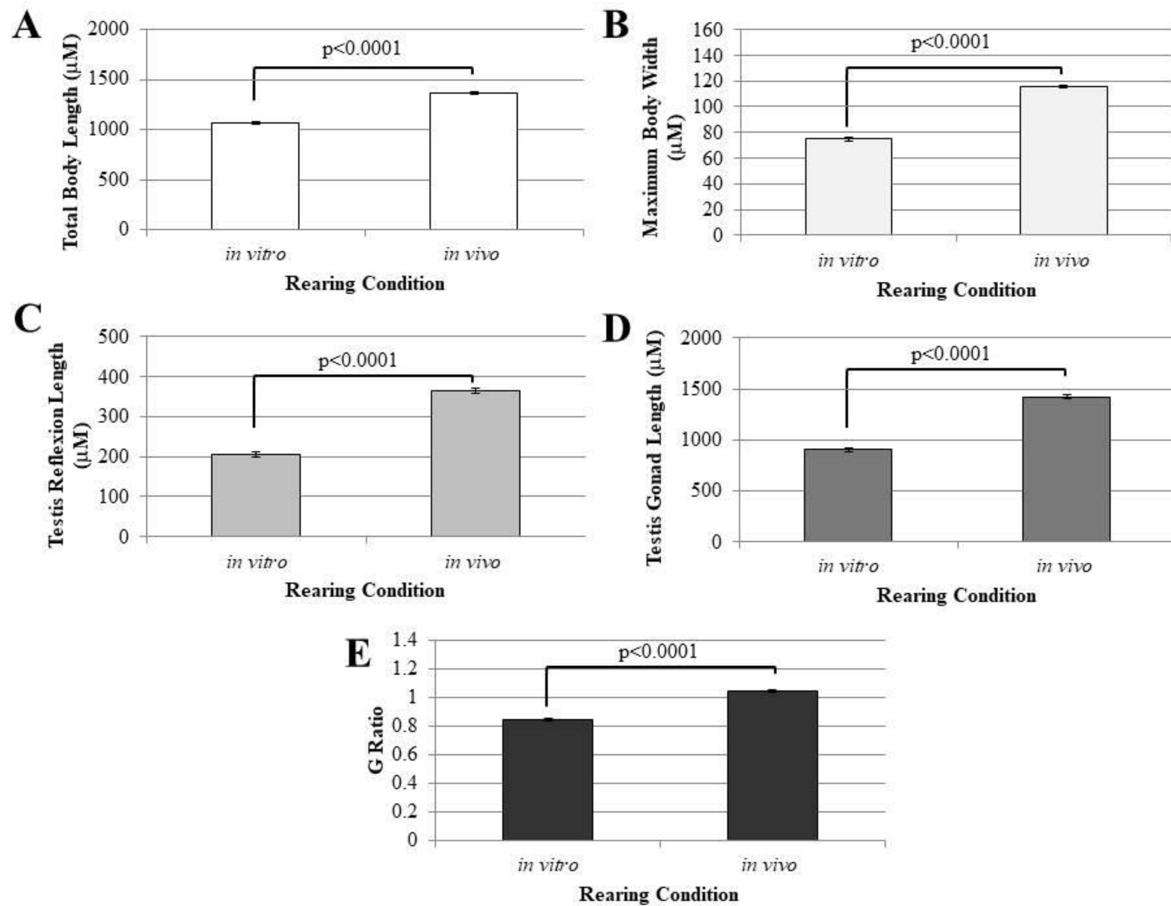


A. Total body length, **B.** Maximum body width, **C.** Total gonad length, and **D.** G (gonad length/body length). A mixed-effects model with $\alpha=0.05$ was run treating replicate as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Student's t-test analysis was performed.

Figure S7. *S. feltiae* males under different rearing conditions

A. Male reared *in vitro* with cognate *X. bovienii*, **B.** Male colonized with cognate *X. bovienii* reared *in vivo*

Figure S8. Comparison of morphometric traits measured of *S. feltiae* males reared *in vitro* with their cognate symbiont and reared *in vivo*



A. Total body length, **B.** Maximum body width, **C.** Testis reflexion, **D.** Total gonad length and **E.** G (gonad length/body length). A mixed-effects model with α=0.05 was run treating replicate as a random effect and bacterial condition as a fixed effect. Least square means and standard errors from the mixed-effects ANOVA model are presented. A *post hoc* Student's t-test analysis was performed.

CHAPTER 3: INFLUENCE OF *XENORHABDUS* SYMBIONTS ON ASCAROSIDE (PHEROMONE) SIGNALING IN *STEINERNEMA* NEMATODES

Abstract

In this study, we assessed the effect of symbiotic (cognate and non-cognate) and non-symbiotic bacteria on ascaroside production in two *Steinernema* spp.: *S. carpocapsae* All strain and *S. feltiae* SN strain. Each nematode species was reared under three bacterial scenarios: a) cognate symbiotic, b) non-cognate symbiotic strain, and c) non-cognate symbiotic species. Our results showed *S. carpocapsae* produced five quantifiable ascarosides: asc-C5, asc-C6, asc-C7, asc-C11 and a novel ascaroside-like molecule, glucoside-1 [GLC-1]. With the exception of GLC-1, no significant differences were observed in the ascaroside profile of *S. carpocapsae* when reared under the different bacterial conditions. Specifically, significantly lower production of GLC-1 was observed when *S. carpocapsae* was reared with a non-cognate symbiotic strain, *Xenorhabdus nematophila* All strain. In *S. feltiae* only three molecules were quantifiable: asc-C5, asc-C7 and asc-C11. Similar to *S. carpocapsae*, bacterial conditions did not significantly affect the quantity of the secreted ascarosides, except for one molecule. Asc-11 was not detected when the nematodes were reared with a non-cognate symbiotic species, *Xenorhabdus nematophila* All strain. We conclude that *Xenorhabdus*, as a food source, has an indirect effect on the quantity and quality of ascaroside production by *Steinernema* nematodes.

1. Introduction

Ascarosides are small signaling molecules secreted by nematodes that function as pheromones. Several studies have shown that these molecules influence various aspects of nematode behavior and development (Jeong et al., 2005; Butcher et al. 2007; Srinivasan et al., 2008; Srinivasan et al., 2012). Ascarosides were first described in the parasitic Ascardia nematodes as molecules consisting of fatty acids and a dideoxysugar ascarylose base (Ludewig and Schroeder, 2013). However, they have since been discovered to exist across the Nematoda as common signaling molecule (Choe et al., 2012a).

A significant amount of research has been done in the model organism *Caenorhabditis elegans*. Most studies have focused on the ability of ascarosides to induce the formation of the dauer (non-feeding, environmentally-resistant) stage, (Golden and Riddle, 1982; Jeong et al., 2005; Srinivasan et al., 2008; Butcher et al., 2007; Butcher et al., 2008). For example, Jeong et al. (2005) identified asc-C7, or daumone, an ascaroside that induced the dauer stage.

Further research has shown that asc-C7 is not the only ascaroside responsible for inducing dauer formation. Butcher et al. (2007) showed that two other molecules, asc-C6-MK and asc-ΔC9, are part of the dauer pheromone. Additionally, another molecule, asc-ωC3, was found to act synergistically with other ascaroside components of the dauer pheromone (Butcher et al., 2008). Furthermore, asc-ΔC7-PABA (Pungaliya et al., 2009), and an indole ascaroside known as IC-asc-C5 (Butcher et al., 2009), were shown to be components of the dauer pheromone.

Ascarosides have also been demonstrated to influence social behavior (Srinivasan et al., 2012), and mate attraction (Srinivasan et al., 2008). In regards to social behavior, Srinivasan et al. (2012) demonstrated that indole ascarosides served as aggregation and attraction signals in

both social and solitary *C. elegans* strains. Additionally, Srinivasan et al. (2008) elucidated the mating signal utilized by *C. elegans* hermaphrodites to attract males, a synergistic blend of asc-C6-MK, asc-ΔC9 and Glc-asc-C6-MK. Interestingly, this blend only attracted males at low concentrations (Srinivasan et al., 2008). Additionally, Pungaliya et al. (2009) discovered that another ascaroside, asc-ΔC7-PABA, functions as a potent male attractant.

Several studies have demonstrated that ascaroside signaling is dependent on bacterial food supply. Golden and Riddle (1984) demonstrated that in *C. elegans,* food supply serves as a signal that acts antagonistically to the dauer pheromone by enhancing the recovery of nematodes from the dauer state to their last larval stage. Additionally, a study by Kaplan et al. (2011) demonstrated that *C. elegans* reared with an abundance of *Escherichia coli* elaborated more ascarosides than those reared in starvation conditions.

Ascarosides are highly conserved across the phylum Nematoda as a common signaling system; however, each species has a unique ascaroside profile (Choe et al., 2012a). Only recently has the research expanded to entomopathogenic nematodes (EPNs). For example, Kaplan et al. (2012) showed asc-C5 is commonly found in both *Steinernema* and *Heterorhabditis* species. Additionally, asc-C5 was shown to play a role in the dispersal of *S. feltiae* IJs (Kaplan et al., 2012). Noguez et al. (2012) found that ascaroside asc-C11-EA inhibits recovery of *Heterorhabditis* IJs into the fourth juvenile stage.

Entomopathogenic nematodes have an obligate mutualistic relationship with *Xenorhabdus* bacteria (Boemare et al., 1993; Boemare, 2002). *Xenorhabdus* symbionts facilitate many parts, including acting as a food source for the developing nematodes once inside an insect cadaver, allowing the nematodes to recover from their IJ stage and grow and reproduce. Studies have demonstrated deleterious effects when a nematode does not pair with its cognate symbiont.

Specifically, nematodes are less fit when they pair with non-cognate symbionts (Sicard et al., 2004; Murfin et al., 2015; McMullen et al., 2017). However, until now, no research has been done to elucidate the role that *Xenorhabdus* may have on ascaroside production of EPNs. Since these bacterial symbionts are key players in *Steinernema* fitness, we hypothesize that *Xenorhabdus* partners have an effect on the production of ascarosides in *Steinernema* nematodes. The experiments outlined in this study aimed at addressing this hypothesis.

2. Materials and Methods

Nematode and bacterial rearing conditions as well as experimental setup followed procedures described in Chapter 2 [Sections 2.1-2.2 and 2.4-2.5] with the exception of the size of the Petri dishes and the amount of bacterial culture plated. Instead, 5 cm diameter Petri dishes containing were utilized, along with 100 μL of bacterial culture to make the lawns. Additionally, the experimental setup was completed twice in a day to increase the yield of adults. Figure 1 shows a schematic representation of the experimental setup.

2.1 Collection of ascarosides

The timeframe for collection of adults varied between 4-6 days, depending on the bacterial treatment. For ascaroside quantification, adult nematodes were harvested under sterile conditions in a laminar flow hood. Briefly, nematodes were rinsed off the plates with Ringer's solution and collected in a 15-mL centrifuge tube (BD Falcon®). Plates from the same bacterial treatment were combined into one pool.

Nematodes were allowed to settle to the bottom of the tube and the supernatant was removed. Nematodes were rinsed 2-3 times until all debris was removed. An additional final rinse was done with 5-6 mL of Ringer's solution.

The nematode solution was homogenized by pipetting the suspension up and down. Three to six 100 µL aliquots were removed from the pool and dispensed onto a hemocytometer to quantify the total number of adults. The counts were utilized to determine the total number of adults in the sample. Triton-X was utilized as a surfactant to prevent nematodes from sticking to the pipette tips. Aliquots of 1000 *S. carpocapsae* adults were dispensed into new 15 mL centrifuge tubes (BD Falcon®). For *S. feltiae*, due to the lower adult yield, aliquots of 300 adults collected from the XbfSN and Xbp conditions were collected and dispensed into new 15 mL centrifuge tubes. Due to the extremely low adult yields when *S. feltiae* was reared with XnAll, the total amount of adults from this condition was pooled (100 or less). The supernatant of each sample was removed and the adults were suspended in 1 mL of Ringer's solution to soak.

The centrifuge tubes were incubated for 3 h while shaking at 180 rpm at 28ºC ± 1ºC. The supernatant from the samples were collected and placed into 1.5 mL microcentrifuge tubes. The samples were centrifuged for 5 min at 2000 rpm to allow any eggs females may have released to pellet. The supernatant was transferred to a 2.0 mL microcentrifuge tube and stored at -80 ºC until lyophilization.

2.2 Analysis of ascarosides

The following protocol was provided by Dr. Butcher's Lab (University of Florida). Lyophilized samples were extracted with 800 µL methanol via sonication and vortex. The samples were centrifuged at 13000 rpm for 5 min., and the supernatant was transferred to a new

1.5 mL microcentrifuge tube and dried in the speedvac for 1 hour. Following drying, 50 μL of methanol was added to extract ascarosides via sonicated and vortex. The microcentrifuge tubes were centrifuged again at 13000 rpm for 5 min., and 10 μL of the supernatant was injected into liquid chromatography mass spectroscopy (LC-MS).

LC-MS analysis was performed on a Phenomenex Luna 5 μm C18 100A (100 x 4.6 mm) column attached to an Agilent 1260 infinity binary pump and Agilent 6130 single quad mass spectrometer with API-ES source, operating in dual negative/positive selected-ion monitoring (SIM) mode. A water (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid) solvent gradient was used, holding at 5% acetonitrile for 5 min., ramping to 60% acetonitrile over 15 min., ramping to 100% acetonitrile, and holding at 100% acetonitrile for 4 min.. Ascarosides were detected by LC-MS using the [M-H] ion. Quantification of ascarosides by LC-MS was calculated by generating a calibration curve utilizing synthetic standard ascarosides. Synthetic ascarosides (asc-C5, asc-C6, asc-C7, asc-C8, asc-C9, asc-C11, asc-C6-MK, IC-asc-C5 and glucoside-1 (GLC-1)) were used as the standards for screening in LC-MS analysis. GLC-1 is a novel molecule with an ascaroside-like structure detected in Dr. Butcher's lab (University of Florida). All LC-MS analyses were performed in Dr. Butcher's lab at the University of Florida.

2.3 Statistical analysis

Statistical analysis of ascarosides was performed in JMP software, version 13 (SAS). A mixed-effects ANOVA model (α=0.05) was considered for analysis. In the mixed-effects model, experimental setup was defined as a random effect and bacterial condition was defined as a fixed effect to account for any possible variability in setup. This was carried out through the fit model function, utilizing a restricted maximum likelihood (REML) method. A *post hoc* least squares

means Tukey's honest significant difference (HSD) analysis was conducted to determine bacterial effects. Each ascaroside molecule was analyzed separately.

3. Results

3.1 S. carpocapsae *ascarosides*

Five ascaroside molecules were quantifiable in *S. carpocapsae* All strain: asc-C5, asc-C6, asc-C7, asc-C11 and glucoside-1 (GLC-1). Overall, Asc-C5 was the most abundantly produced ascaroside molecule, followed by GLC-1. Asc-C11 was minimally produced, with unquantifiable amounts when the nematodes were reared with non-cognate symbionts. An increase in the production of asc-C5 was observed when the nematodes were reared in a non-cognate strain of *X. nematophila* (Anatoliense), ranging from 10.09 pmoles/1,000 adults to 12.32 pmoles/1,000 adults (Fig. 2). However, this increase was not significantly different. Production of asc-C6, asc-C7 and asc-11, was not significantly affected by the bacterial source used to rear the nematodes (Fig. 2). Contrastingly, amounts of GLC-1 significantly decreased from 6.59 pmoles/1,000 adults when *S. carpocapsae* was cultured with cognate XnAll, to 2.55 pmoles/1,000 adults when *S. carpocapsae* was cultured with the non-cognate *X. nematophila* strain, XnAna (Fig. 2). Results from the mixed-effects ANOVA analysis showed there was a statistical difference in GLC-1. These observations were also supported by the Tukey HSD *post hoc* test.

3.2 S. feltiae *ascarosides*

Three ascaroside molecules were quantifiable in *S. feltiae* SN strain*:* asc-C5, asc-C7 and asc-C11. A weak peak with a retention time similar the one observed for GLC-1 was detected in

LC-MS analysis, however further analysis need to be done to corroborate this observation. There were no detectable amounts of asc-C6 in this species.

Similar to *S. carpocapsae,* asc-C5 was the most abundantly produced ascaroside molecule. Although larger quantities of asc-C5 were detected when *S. feltiae* was reared with cognate and non-cognate strains of *X. bovienii*, this increase was not considered significantly different (Fig. 3). Asc-C7 was produced in small amounts (ranging from 5.34 pmol/1,000 adults to 8.09 pmol/1,000 adults) regardless of the rearing condition. Asc-C11 was not detected when *S. feltiae* nematodes were reared with the non-cognate species, *X. nematophila* All strain. This lack of asc-C11 production was considered to be significant (Fig. 3).

4. Discussion

Ascaroside molecules take part in a variety of sex-specific and social behaviors including male attraction, and aggregation in *C. elegans* (Srinivasan et al., 2008; Srinivasan 2012). In this study, we examined the ascaroside profiles of two *Steinernema* spp. when reared in the presence of cognate and non-cognate *Xenorhabdus* species and/or strains.

Our results showed that both the quality and quantity of ascarosides secreted by each of the tested *Steinernema* spp. varied when the nematodes were cultivated on their cognate symbiont or on non-cognate symbionts. For example, *S. feltiae* produced larger amounts of ascarosides that *S. carpocapsae*. However, the diversity of the secreted pheromones in *S. carpocapsae* was higher. While five ascaroside molecules (asc-C5, asc-C6, asc-C7, asc-C11, and GLC-1) were identified in *S. carpocapsae*, only three (asc-C5, asc-C7, and asc-C11) were quantified in *S. feltiae*. In this respect, a study by Choe et al. (2012a) demonstrated that ascaroside profiles are unique for every species. Similarly, the authors reported that *S.*

carpocapsae adults produced asc-C6, but this molecule was undetected in *Steinernema riobrave*, indicating that asc-C6 is specific to only certain *Steinernema* species, which is consistent with our findings.

Bacterial culture conditions differentially affected each *Steinernema* spp. with respect to ascaroside production. For example, non-cognate strain (XnAna) and non-cognate species (XbfSN) enhanced the production of asc-C5 in *S. carpocapsae*. Similarly, non-cognate Xbp strain favored production of this ascaroside in *S. feltiae*. Although interesting, from a statistical stand point, these changes were not significant. While asc-C5 stimulates dispersal of *Steinernema* IJs (Kaplan et al., 2012), its role in the behavior of adult nematodes is still undetermined. In relation to this, results from the research outlined in Chapter 2 also revealed that body size of first-generation males of *S. carpocapsae* increased when reared with this non-cognate XnAna strain. However, it is not clear if body size is a factor that contributes to a larger production of ascarosides by adult nematodes.

Each *Steinernema* spp. exhibited significant differences in one ascaroside molecule when reared with different *Xenorhabdus* symbionts. Specifically, *S. feltiae* exhibited a significant decrease in asc-C11 when reared with a non-cognate symbiotic species (XnAll). Interestingly, asc-C11 is a major ascaroside in plant-parasitic nematodes and induces plant defenses (Manosalva et al., 2015), but its role is not well-defined in entomopathogenic nematodes. Choe et al. (2012a) demonstrated that entomopathogenic nematodes produce this ascaroside in small concentrations. Additionally, Noguez et al. (2012) demonstrated that a chemically modified version of this ascarosides, ascaroside C11 ethanolamine (asc-C11-EA) induces IJ formation in the entomopathogenic nematode *Heterorhabditis bacteriophora*. Since this ascaroside is not

well characterized in the *Steinernema* genera, it is unknown why this ascaroside is not produced when *S. feltiae* is reared with non-cognate species XnAll.

The production of GLC-1 was significantly reduced when *S. carpocapsae* was reared with a non-cognate symbiotic XnAna strain. Although this reduction was determined as significant by the *post hoc* Tukey's HSD test, there was considerable variation in GLC-1. GLC-1 is a novel molecule, and its function is currently being determined. A larger sample size may have led to difference statistical conclusions.

Preliminary studies were conducted to determine if ascaroside biosynthesis is sex-specific in entomopathogenic nematodes. Our preliminary experiments showed that *S. carpocapsae* females produced more ascarosides than males (Fig. S1). This observation is verified in early studies by Fulk and Shorb (1976), which reported that females of *Ascaridia galli* produced higher concentrations of ascaroside molecules than males. Similarly, Izrayelit et al. (2010) demonstrated that ascaroside biosynthesis is sex specific in *C. elegans*. However, further research utilizing other entomopathogenic nematode species needs to be conducted.

Altogether, this study showed that *Xenorhabdus* as a food source plays an important role in the type and amount of ascarosides secreted by *Steinernema* spp. In relation to this, food has been demonstrated in several studies to influence ascaroside production in *C. elegans* (Golden and Riddle, 1984; Kaplan et al., 2011). The results from this study were species-specific, and further research should target other *Steinernema* spp. and adult generations. Additionally, further research is needed to elucidate if body size, and other morphometric parameters affected by *Xenorhabdus* symbionts, are factors that influence ascaroside production by adult nematodes. Understanding the interaction of ascaroside production and food supply will make a significant

contribution not only to the unravelling of the life cycle of these entomopathogens, but will also contribute to the optimization of culturing methods for mass production systems.

Figure 1. Schematic representation of ascaroside experimental design

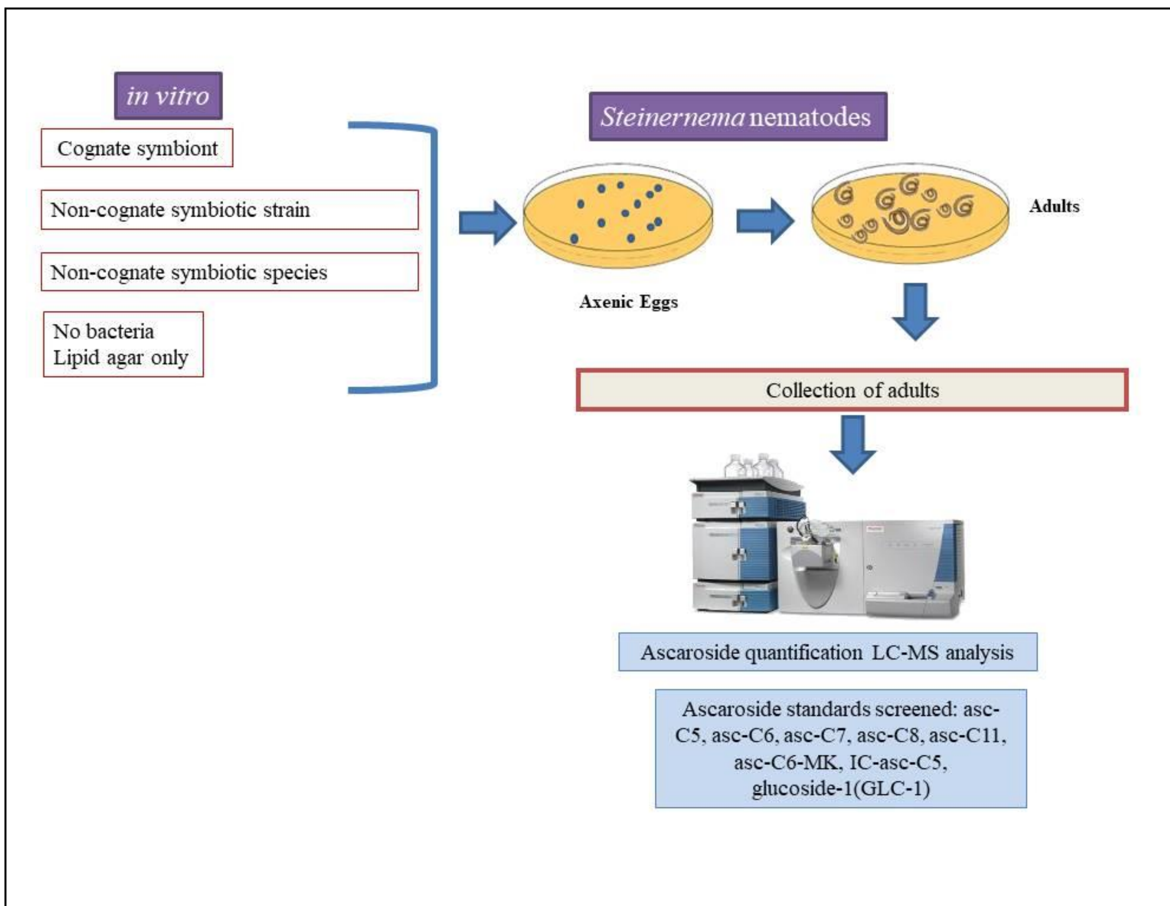
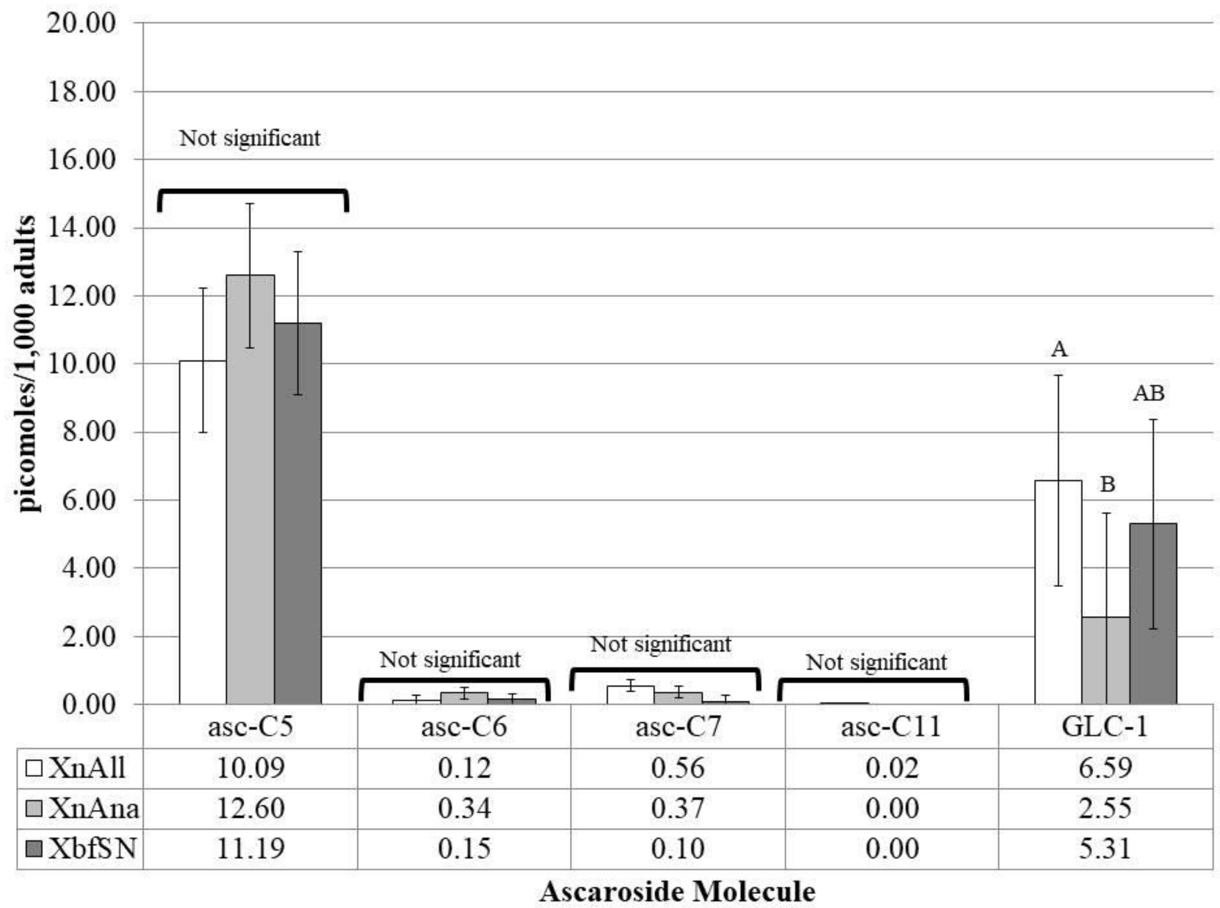


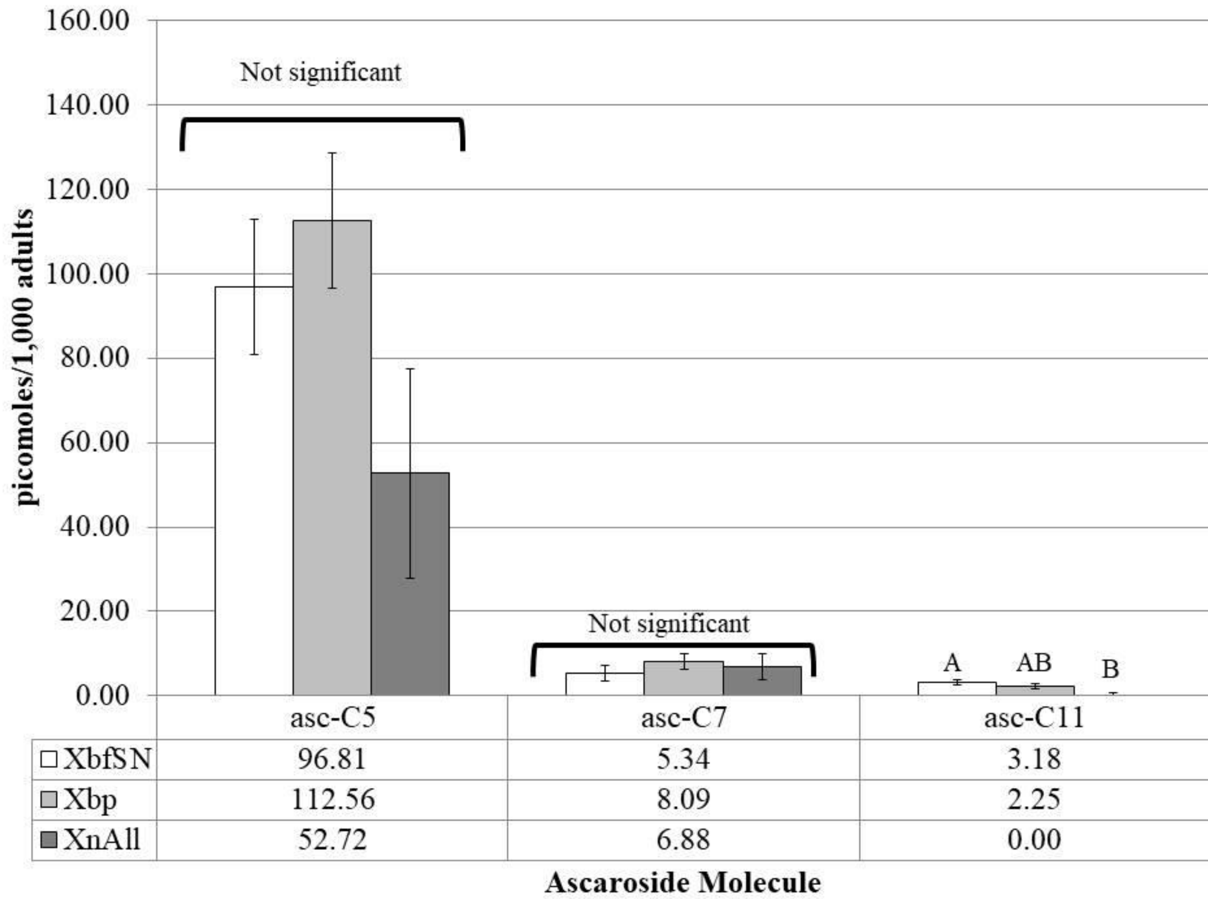
Figure 2. *S. carpocapsae* ascaroside profiles when reared with various *Xenorhabdus* symbionts



	asc-C5	asc-C6	asc-C7	asc-C11	GLC-1
XnAll	10.09	0.12	0.56	0.02	6.59
XnAna	12.60	0.34	0.37	0.00	2.55
XbfSN	11.19	0.15	0.10	0.00	5.31

A mixed-effects ANOVA model with α=0.05 was run treating experimental setup as a random effect and bacterial condition as a fixed effect. Least square means and standard error from the model are presented. A *post hoc* Tukey's HSD analysis was performed.

Figure 3: *S. feltiae* ascaroside profiles when reared with various *Xenorhabdus* symbionts

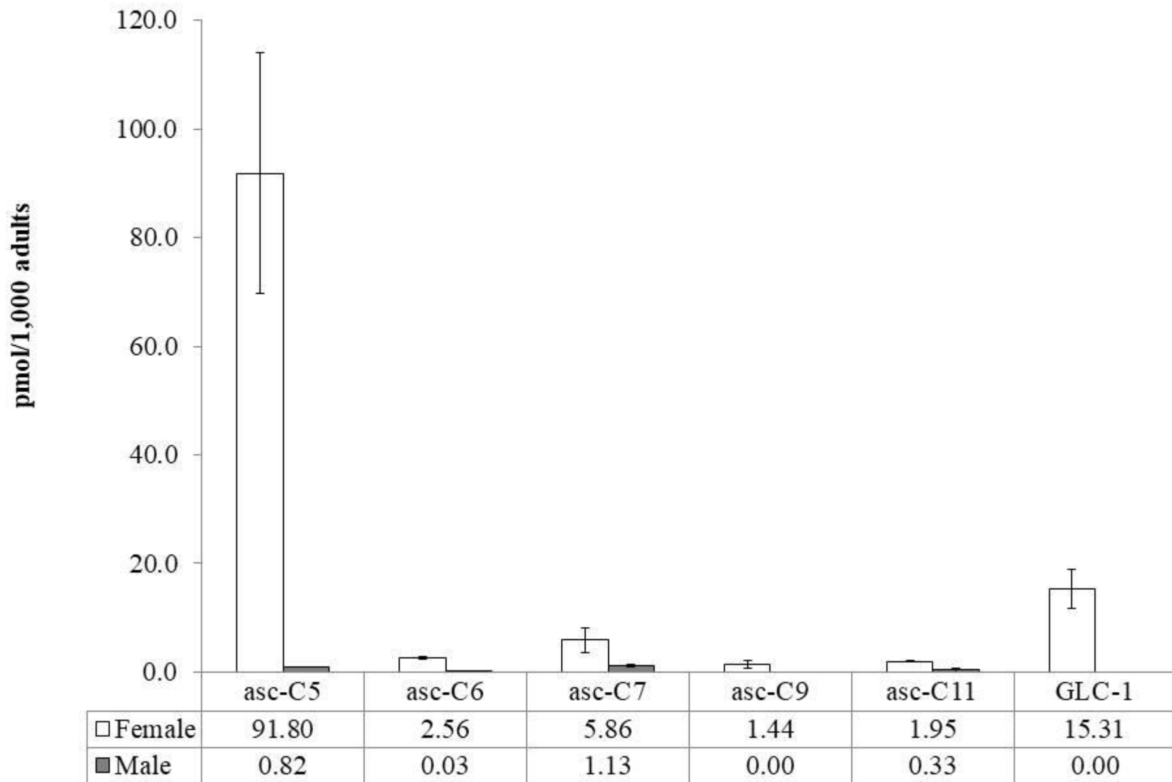


A mixed-effects ANOVA model with α=0.05 was run treating experimental setup as a random effect and bacterial condition as a fixed effect. Least square means and standard error from the model are presented. A *post hoc* Tukey's HSD analysis was performed.

Supplemental Documents

Preliminary experiments were performed to determine if females and males of *Steinernema* nematodes produce different amounts of ascarosides. *S. carpocapsae* All strain nematodes were reared *in vivo* utilizing the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) according to procedures described by Kaya and Stock (1997). Briefly, a nematode suspension of 1.8 mL (approximately 1,000-2,000 IJs/mL was pipetted onto a 10cm Petri dish lined with two discs of filter paper (Whatman #1). Approximately 10-12 *G. mellonella* larvae were added to the plate, which was covered and stored at 25 ºC in the dark. Approximately 3.5-4 days later, cadavers were dissected in Ringer's buffer, and 300 females and males were collected. Collection and analysis of ascarosides follows procedures outlined in the sections 2.5 and 2.6 of the materials and methods section of this chapter. This preliminary study was repeated twice.

Figure S1. *S. carpocapsae* female and male differential ascaroside production under *in vivo* conditions



Means are presented. Standard error is represented by the error bars.

REFERENCES

Akhurst, R.J., 1982. Antibiotic activity of *Xenorhabdus* spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. J. Gen. Microbiol. 128, 3061-3065.

Akhurst, R.J., 1983. Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. Int. J. Syst. Bacteriol. 33, 38-45.

Alsaiyah, M.A.M., Ebssa, L., Zenner, A., O'Callaghan, K.M., Griffin, C.T., 2009. Sex ratios and sex-biased infection behavior in the entomopathogenic nematode genus *Steinernema.* Int. J. Parasitol. 39, 725-734.

Anderson, R.C., 2000. Nematode parasites of vertebrates: Their development and transmission. CABI Publishing, Wallingford, UK.

Bartley, J.P., Bennett, E.A., Darben, P.A., 1996. Structure of the ascarosides from *Ascaris suum*. J. Nat. Prod. 59, 921-926.

Bedding, R.A., 1967. Parasitic and free-living cycles in entomogenous nematodes of the genus *Deladenus*. Nature. 214, 174-175.

Bernard, E.C., 1992. Soil nematode biodiversity. Biol. Fert. Soils. 14, 99-103.

Bird, A.F., Akhurst R.J., 1983. The nature of the intestinal vesicle in nematodes of the family Steinernematidae. Int. J. Parasitol. 16, 511-518.

Blaxter, M., Bird, D., 1997. Parasitic Nematodes, in: Riddle, D.L., Blumenthal, T., Meyer, B.J., Priess, J.R. (Eds.), *C. elegans* II. https://www.ncbi.nlm.nih.gov/books/NBK20125.

Boemare, N.E., 2002. Biology, taxonomy and systematics of *Photorhabdus* and *Xenorhabdus,* in: Gaugler, R. (Ed.), Entomopathogenic Nematology. CABI Publishing, Wallingford, UK, pp. 35-56.

Boemare, N.E., Akhurst, R.J., Mourant, R.G., 1993. DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. Int. J. Syst. Bacteriol. 43, 249-255.

Bosshardt, S.C., McCall, J.W., Coleman, S.H., Jones, K.L., Petit, T.A., Klei, T.L., 1993. Prophylactic activity of tetracycline against *Brugia pahangi* infection in Jirds (*Meriones unuiculatus*). J. Parasitol. 79, 775-777.

Butcher, R.A., Fujita, M., Schroeder, F.C., Clardy, J., 2007. Small-molecule pheromones that control dauer development in *C. elegans*. Nat. Chem. Biol. 3, 420-422.

Butcher, R.A., Ragains, J.R., Clardy, J., 2009. An indole-containing dauer pheromone component with unusual dauer inhibitory activity at higher concentrations. Org. Lett. 11, 3100-3103.

Butcher, R.A., Ragains, J.R., Kim, E., Clardy, J., 2008. A potent dauer pheromone component in *C. elegans* that acts synergistically with other components. Curr Issues 105, 14288-14292.

Campos-Herrera, R., Babercheck, M., Hoy, C.W., Stock, S.P., 2012. Entomopathogenic nematodes as a model system for advancing the frontiers of ecology. J. Nematol. 22, 162-76.

Campos-Herrera, R., Guitérrez, C., 2014. *Steinernema feltiae* intraspecific variability: infection dynamics and sex ratio. J. Nematol. 46, 35-43.

Chapuis, É., Arnal, A., Ferdy, J.-B., 2012. Trade-offs shape the evolution of the vector-borne insect pathogen *Xenorhabdus nematophila*. Proc. R. Soc. B. 279, 2672-2680.

Chapuis, E., Emelianoff, V., Paulmier, V., Le Brun, N., Pagès, S., Sicard, M., Ferdy, J.-B. 2009. Manifold aspects of specificity in a nematode-bacterium mutualism. J. Evol. Biol. 22, 2104-2117.

Chen, G., Dunphy, G.B., Webster, J.M., 1994. Antifungal activity of two *Xenorhabdus* species and *Photorhabdus luminescens* of *Heterorhabditis megidis.* Biol. Control. 4, 157-162.

Choe, A., Chuman, T., von Reuss, S.H., Dossey, A.T., Yim, J.J., Ajredini, R., Kolawa, A.A., Kaplan, F., Alborn, H.T., Teal, P.E.A., Schroeder, F.C., Sternberg, P.Q., Edison, A.S., 2012b. Sex-specific mating pheromones in the nematode *Panagrellus redivivus*. Proc. Natl. Acad. Sci. U.S.A. 109, 20949-20954.

Choe, A., von Reuss, S.H., Kogan, D., Gasser, R.B., Platzer, E.G., Schroeder, F.C., Sternberg, P.W., 2012a. Ascaroside signaling is widely conserved among nematodes. Curr. Biol. 22, 772-780.

Costa, H.S., Enneberry, T.J., Toscano, N.C., 1997. Effects of antibacterial materials on *Bemisia argentifoli* (Homoptera: Aleyrodidae) oviposition, growth, survival and sex ratio. J. Econ. Entomol. 90, 333-339.

Costa, H.S., Ullman D.E., Johnson, M.W., Tabashnik, B.E., 1993. Antibiotic oxytetracycline interferes with *Bemisia tabaci* (Homoptera: Aleyrodidae) oviposition, development, and ability to induce squash silverleaf. Ann. Entomol. Soc. Am. 86, 740-748.

Courtney, W.D., Polley, D., Miller, V.L., 1955. T.A.F., an improved fixative in nematode technique. Pl. Dis. Repir. 39, 570-571.

Cowles, K.N., Goodrich-Blair, N., 2008. The *Xenorhabdus nematophila nilABC* genes confer the ability of *Xenorhabdus* spp. to colonize *Steinernema carpocapsae* nematodes. J. Bacteriol. 190, 4121-4128.

De Ley, P., 2006. A quick tour of nematode diversity and the backbone of nematode phylogeny. WormBook: The Online Review of *C. elegans* Biology. http://www.ncbi.nlm.nih.gov/books/NBK19684.

Dillman, A.R., Chaston, J.M., Byron, J.A., Ciche, T.A., Goodrich-Blair, H. Stock, S.P., Sternberg, P.W., 2012. An entomopathogenic nematode by any other name. PLoS Pathog. 8, e1002527.

Eleftherianos, I., Joyce, S., ffrench-Constant, R.H., Clarke, D.F., Reynolds, S.E., 2010. Probing the tri-trophic interaction between insects, nematodes and *Photorhabdus*. Parasitology 137, 1695-1706.

Ettema, C.H., 1998. Soil nematode diversity: Species coexistence and ecosystem function. J. Nematol. 30, 159-169.

Fairbain, D., 1957. The biochemistry of *Ascaris*. Exp. Parasitol. 6, 491.554.

Fischer-Le Saux, M., Arteaga-Hernández, E., Mráček, Z., Boemare, N.E., 1999. The bacterial symbiont *Xenorhabdus poinarii* (Enterobacteriaceae) is harbored by two phylogenetic related host nematodes: the entomopathogenic species *Steinernema cubanum* and *Steinernema glaseri* (Nematoda: Steinernematidae). FEMS Microbiol. Ecol. 29, 149-157.

Flury, F., 1912. On the chemistry and toxicology of ascarides. Arch. Exp. Path. Pharmak. 67, 275-392.

Foor, W.E., 1967. Ultrastructural aspects of oocyte development and shell formation in *Ascaris lumbricoides.* J. Parasitol. 53, 1245-1261.

Fulk, W.K., Shorb, M.S., 1971. Lipids of male and female *Ascaridia galli* at different ages. J. Parasitol. 57, 840-844.

Gaugler, R, Kaya, H., 1993. Entomopathogenic nematodes. Annu. Rev. Entomol. 38, 181-206.

Golden, J.W., Riddle, D.L., 1982. A pheromone influences larval development in the nematode *Carnorhabditis elegans*. Science 218, 578-580.

Golden, J.W., Riddle, D.L., 1984. The *Carnorhabditis elegans* dauer larva: developmental effects of pheromone, food, and temperature. Dev. Biol. 102, 368-278.

Grewal, P.S., Ehlers, R.U., Shapiro-Ilan, D.I., 2005. Nematodes as Biological Agents. CABI Publishing, Wallingford, UK.

Griffin, C.T., O'Callaghan, K.M., Dix, I., 2001. A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution among entomopathogenic nematodes? Parasitology 122, 181-186.

Heungens, K., Cowles, C.E., Goodrich-Blair, H., 2002. Identification of *Xenorhabdus nematophila* genes required for mutualistic colonization of *Steinernema carpocapsae* nematodes. Mol. Micobiol. 45, 1337-1353.

Hoerauf , A., Nissen-Pahle, K., Schmetz, C., Henkle-Duhrsen, K., Blaxter, M.L., Büttner, D.W., Gallin, M.Y., Al-Quaoud, K.M., Lucuis, R., Fleisher, B., 1999. Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. J. Clin. Invest. 103, 11-18.

Hoerauf, A., Volkmann, L., Nissen-Pahle, K., Schmetz, C., Autenrieth, I., Büttner, D.W., Fleischer, B., 2000. Targeting of *Wolbachia* endobacteria in *Litomosoides sigmodontis* comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxacin. Trop. Med. Intl. Health. 5, 275-279.

Izrayelit, Y., Srinivasan, J., Campbell, S.L., Jo, Y. von Reuss, S.H., Genoff, M.C., Sternberg P.W., Schroeder, F.C., 2012. Targeted metabolomics reveals a male pheromone and sex-specific ascaroside biosynthesis in *Caenorhabditis elegans.* ACS Chem. Biol. 7, 1321-1325.

Jeong, P.Y., Jung, M., Yim, Y.H., Kim, H., Park, M., Hong, E., Lee, W., Kim, Y.H., Kim, K., Paik, Y.K., 2005. Chemical structure and biological activity of the *Caenorhabditis elegans* dauer-inducing pheromone. Nature 433, 541-545.

Jezyk, P.F., Fairbairn, D., 1967. Ascarosides and ascaroside esters in *Ascaris lumbricoides* (Nematoda). Comp. Biochem, Physiol. 23, 691-705.

Kaplan, F., Alborn, H.T., von Reuss, S.H., Ajredini, R., Ali, J.G., Akyazi, F., Stellinski, L.L., Edison, A.S., Schroeder, F.C., Teal, P.E., 2012. Interspecific nematode signals regulate dispersal behavior. PLoS ONE 7, e38735.

Kaplan, F., Srinivasan, J., Mahanti, P., Ajredini, R., Durak, O., Nimalendran, R., Sternberg, P.W., Teal, P.E.A., Schroeder, F.C., Edison, A.S., Alborn, H.T., 2011. Ascaroside expression in *Caenorhabditis elegans* is strongly dependent on diet and developmental stage. PLoS ONE 6, e17804.

Kaya, H.K., Stock, S.P., 1997. Techniques in insect nematology, in: Lacey, L.A. (Ed.), Manual of techniques in invertebrate pathology, 1st ed. Academic Press, San Diego, CA, pp. 281-324.

Knopp, S., Steinmann, P.,Hatz, C., Keiser, J., Utzinger, J., 2012. Nematode infections: filariases. Infect. Dis. Clin. N. Am. 26, 359-381.

Kozek, W.J., 1977. Transovarially-transmitted intracellular microorganisms in adult and larval stages of *Brugia malayi.* J. Paraistol. 63, 992-1000.

Kozek, W.J., 2005. What is new in the *Wolbachia/Dirofilaria* interaction? Vet. Paraistol. 133, 127-32.

Lacey, L.A., Georgis, R., 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. J. Nematol. 44, 218-25.

Lambshed, P.J.D., Boucher, G., 2003. Marine nematode deep-sea biodiversity- hyperdiverse or hype? J. Biogeogr. 30, 475-485.

Landmann, F., Voronin, D., Sullivan, W., Taylor, M.J., 2011. Anti-filarial activity of antibiotic therapy is due to extensive apoptosis after *Wolbachia* depletion from filarial nematodes. PLoS Pathog. 7, e1002351.

Lee, M.M., Stock, S.P., 2010. A multi-locus approach to assessing co-evolutionary relationships between *Steinernema* spp. (Nematoda: Steinernematidae) and their bacterial symbionts *Xenorhabdus* spp. (γ-Proteobacteria: Enterobacteriaceae). Syst. Paraistol. 77, 1-12.

Ludewig, A.H., Schroeder, F.C., 2013. Ascaroside signaling in *C. elegans.* Wormbook, 1-22.

Manosalva, P., Manohar, M., von Reuss, S.H.., Chen, S., Koch, A., Kaplan, F., Choe, A., Micikas, J., Wang, X., Kogel, K-H., Sternberg, P.W., Williamson, V.M., Schroeder, F.C., Klessig D.F., 2015. Conserved nematode signaling molecules elicit plant defenses and pathogen resistance. Nat. Commun. 6. doi:10.1038/ncomms8795.

Martens, E.C., Heungens, K., Goodrich-Blair, H., 2003. Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. J. Bacteriol. 185, 3147-3154.

McMullen II, J.G., Peterson, B.F., Forst, S., Goodrich-Blair, H., Stock, S.P., 2017. Fitness costs of symbiont switching using entomopathogenic nematodes as a model. BMC Evol. Biol. 17, 100.

McMullen II, J.G., Stock, S.P., 2014. *In vivo* and *in vitro* rearing of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae). J. Vis. Exp. e52096.

Miranda, V.A., Navarro, P.D., Davidowitz, G., Bronstein, J., Stock, S.P., 2013. Effect of insect host age and diet on the fitness of the entomopathogenic nematode-bacteria mutualism. Symbiosis 61, 145-153.

Montalvo-Katz, S., Huang, H., Appel, M.D., Berg, M., Shapira, M., 2013. Association with soil bacteria enhances p38-dependent infection resistance in *Caenorhabditis elegans.* Infect. Immun. 81, 514-520.

Montgomery, M.K., McFall-Ngai, M., 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. Development 120, 1719-1729.

Murfin, K.E., Lee, M.M., Klassen, J.L., McDonald, B.R., Larget, B., Forst, S., Stock, S.P. Currie, C.R., Goodrich-Blair, H., 2015. *Xenorhabdus bovienii* strain diversity impacts coevolution and symbiotic maintenance with *Steinernema* spp. nematode hosts. mBio. 6:e00076-15.

Noguez, J.H., Conner, E.S., Zhou, Y., Ciche, T.A., Ragains, J.R., Butcher, R.A., 2012. A novel ascaroside controls the parasitic life cycle of entomopathogenic nematode *Heterorhabditis bacteriophora*. ACS Chem. Biol. 7, 961-966.

Nguyen, K.B., Hunt, D.J., Mráček, Z., 2007. Steinernematidae: species descriptions, in: Nguyen, K., Hunt, D.J. (Eds)., Nematology Monographs and Perspective Volume 5: Entomopathogenic Nematodes: Systematics, Phylogeny, and Bacterial Symbionts. Koninklijke Brill BV ,Leiden, Netherlands, pp. 121-609.

Peterson, J.J., 1985. Nematodes as biological control agents: part I. Mermithidae. Adv. Parasitol. 24, 307-344.

Platt, H.M., 1994. Forward, in: Lorenzen, S. (Ed.), Phylogenetic Systematics of Free-Living Nematodes. The Ray Society, London, UK, pp. i-ii

Poinar Jr., G.O., 1983. The natural history of nematodes. Prentice-Hall, Inc., Englewood Cliffs, NJ, pp. 1-323.

Poinar Jr., G.O., 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae, in: Gaugler, R., Kaya, H.K. (Eds)., Entomopathogenic Nematodes in Biologcal Control. CRC Press, Florida, pp. 23-61.

Poinar Jr., G.O., Thomas, G.M., 1966. Significance of *Achromobacter nematophilus* Poinar and Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136 (Neoaplectana so. Steinernematidae). Parasitology 56, 385-90.

Pungaliya, C., Srinivasan, J., Fox, B.W., Malik, R.U., Ludewig, A.H., Sternberg, P.W., Schroeder, F.C., 2009. A shortcut to identifying small molecule signals that regulate behavior and development in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U.S.A. 106, 7708-7713.

Rao, R., Well, G.J., 2002. In vitro effects of antibiotics on *Brugia malayi* worm survival and reproduction. J. Parasitol. 88, 605-611.

San-Blas, E., Gowen, S.R., Pembroke, B., 2008. *Steinernema feltiae:* ammonia triggers the emergence of their infective juveniles. Exp. Parasitol. 119, 180-185.

Sicard, M., Ferdy J.-B., Pagès, S., Le Brun, N., Godelle, B., Boemare, N., Moulia, C., 2004. When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). J. Evol. Biol. 17, 985-993.

Sicard, M., Le Brun, N., Pagès, S., Godelle, B., Boemare, N.E., Moulia, C., 2003. Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interaction. Parasitol. Res. 91, 520-524.

Simon, J.M., Sternberg, P.W., 2002. Evidence of a mate-finding cure in the hermaphrodite nematode *Caenorhabditis elegans.* Proc. Natl. Acad. Sci. U.S.A. 99, 1598-1603.

Snyder, H., Stock, S.P., Kim, S.K., Flores-Lara, Y., Forst, S., 2007. New insights into the colonization and release processes of *Xenorhabdus nematophila* and the morphology and ultrastructure of the bacterial receptacle of its nematode hosts, *Steinernema carpocapsae*. Appl. Environ. Microbiol. 73, 5338-5346.

Srinivasan, J., Kaplan, F., Ajerdini, R., Zachariah, C., Alborn, H.T., Teal, P.E., Malik, R.U., Edison, A.S., Sternberg, P.W., Schroeder, F.C., 2008. A blend of small molecules regulates both mating and development in *Caenorhabditis elegans*. Nature 454, 1115-1118.

Srinivasan, J., von Reuss, S.H., Bose, N., Zaslaver, A., Mahanti, P., Ho, M.C., O'Doherty, O.G., Edison, A.S., Sternberg, P.W., Schroeder, F.C., 2012. A modular library of small molecule signals regulate social behavior in *Caenorhabditis elegans*. PLoS Biol. 10, e1001237.

Stock, S.P., 2005. Insect-parasitic nematodes: from lab curiosities to model organisms. J. Invertebr. Pathol. 89, 57-66.

Stock, S.P., 2015. Diversity, biology and evolutionary relationships, in: Camps-Herrera, R. (Ed.), Nematode Pathogenesis of Insects and Other Pests-Ecology and Applied Technologies for Sustainable Plant and Crop Protection. Springer International Publishing, Switzerland, pp. 3-27.

Stock, S.P., Goodrich-Blair, 2008. Entomopathogenic nematodes and their bacterial symbionts: the inside out of a mutualistic association. Symbiosis 46, 65-75.

Stock, S.P. Goodrich-Blair, H., 2012. Nematode parasites, pathogens and associates of insects and invertebrates of economic importance, in: Lacey, L.A. (Ed.), Manuel of Techniques in Invertebrate Pathology, 2nd ed. Academic Press, San Diego, CA, pp. 373-426.

Stock, S.P., Griffin, C.T., Chaerani, R., 2004. Morphological and molecular characterization of *Steinernema hermaphroditum* n. sp. (Nematoda: Steinernematidae), an entomopathogenic nematode from Indonesia, and its phylogenetic relationships with other members of the genus. Nematology 6, 401-412.

Taylor, M.J., Bandi, C., Hoerauf, A., 2005. *Wolbachia* bacterial endosymbionts of filarial nematodes. Adv. Parasitol. 60, 245-84.

von Reuss, S.H., Bose, N., Srinivasan, J., Yim, J.J., Judkins, J.C., Sternberg, P.W., Schroder, F.C., 2012. Comparative metabolomics reveals biogenesis of ascarosides, a modular library of small molecule signals in *C. elegans.* J. Am. Chem. Soc. 134, 1817-1824.

Wallace, H.R., 1963. The biology of plant parasitic nematodes. CABI Publishing, London, pp. 280.

Weinstein, S.B., Lafferty, K.D., 2015. How do humans affect wildlife nematodes? Trends Parasitol. 31, 222-227.

Yeates, G.W., 1987. How plants affect nematodes. Adv. Ecol. Res. 17, 61-113.