Morphology of *Stylops advarians* (Strepsiptera) and the Effects of Parasitization on its Host, *Andrena milwaukeensis* (Hymenoptera)

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By

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ABSTRACT

Strepsiptera is an enigmatic order of endoparasitic insects known for the extreme sexual dimorphism of the adults, which results in a unique lifecycle. Adult males are free-living, whereas adult females of most of the order's taxa are neotenic and permanently endoparasitic. *Stylops advarians* Pierce, a parasite of *Andrena milwaukeensis* Graenicher, was collected in central Saskatchewan, Canada and verified to species according to the DNA sequencing analysis of a mitochondrial cytochrome oxidase I. The morphology of the adult male, and the structure of the adult female and first-instar larva, were examined. The adult male was described for the first time from the puparia of a single host. Notable features were the metathoracic scutellum, which reaches close to the prescutum, the antennae, with the fourth antennomere being less than twice as long as the fifth, and the hook-like aedeagus.

Like other *Stylops* species, the adult female of *S. advarians* lacked many adult characteristics, with only her cephalothorax having identifiable features. Impact of the female parasite on her host was examined. Female bees that were stylopized by one or more parasitic females did not have any eggs, nor reproductive organs, within their abdomens, as opposed to non-stylopized bees. Internally within the host gaster, the foregut and partial midgut of bees with one female parasite were shifted away from the parasite. If two or three parasites were present, then the gaster's components of the digestive tract were shifted below the parasites. The crop was heavily reduced in volume when parasitism occurred, but the hindgut was not greatly affected. The female parasites were found to reside above an air sac within their host's gaster.

First-instar larvae have several sensory structures on their head, including eye spots, olfactory pits, and numerous pairs of setae. On the legs, the pro- and mesothoracic tarsi are modified as adhesive structures, whereas those of the metathoracic legs are likely only used for

movement. Similarly, the tips of the caudal filaments likely have an adhesive function, and the coarse hairs (spinulae) covering the thorax and abdomen on both dorsal and ventral surfaces likely help these larvae phoretically attach onto a host. For the first time, first-instars were reported on the surface of a non-stylopized host of *A. milwaukeensis*, as well as inside the crop of a non-stylopized *A. milwaukeensis*, likely as phoronts.

Field-collection data for foraging bees of *A. milwaukeensis* was also recorded to determine seasonal occurrence of stylopization and different parameters of the parasite's life history, including prevalence, intensity, and abundance, within this bee population. From greater than 450 bees taken over three consecutive years (2016–2018), from early May to late June, the prevalence of stylopized bees was found to be around 22% (21–24%). Despite attempts, males were not collected consistently, so their data are not included in this prevalence. The mean intensity of parasite infection was 1.2 (1.167–1.244) and parasite abundance was 0.27 (0.258–-0.276), strongly suggesting that the current relationship of *S. advarians* parasitizing *A. milwaukeensis*, at this study site, is in balance. Stylopized female bees were found to emerge earlier than non-stylopized bees, possibly due to manipulation by the parasite. Mating of the parasites evidently occurred during this emergence, which was around May 2 each year. First-instar larvae began to emerge around May 22, suggesting mating takes place at similar times each year, and that the developmental period of the first-instars is short.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 Introduction to the Order Strepsiptera

The endoparasitic insect order Strepsiptera is best known for its unique and complex life cycle, which is due to extreme sexual dimorphism. Insects parasitized by a strepsipteran are often characterized as being stylopized. In 1793, males of *Xenos vesparum* became the first strepsipteran species to be classified (Rossius, 1793). However, at the time these specimens were classified, they were considered to be parasitic hymenopterans. Later, in 1802, *Stylops melittae* was described (Kirby, 1802), but the previous description of *X. vesparum* was unknown to Kirby. Over a decade later, after Kirby learned of *X. vesparum*, he erected the order Strepsiptera (Kirby, 1813). There have been many debates since the erection of this order about where Strepsiptera should be placed within Insecta, its phylogenetic relationship with other insects, and if Strepsiptera should be considered its own order (Lamarck, 1816; Latrielle, 1818: Swainson and Shuckard, 1840; Bohart, 1941; Crowson, 1954, 1960; Arnett, 1968; Whiting and Wheeler, 1994; Whiting et al., 1997). Recent morphological and molecular analyses suggest this order is a sister group to Coleoptera (Wiegmann et al., 2009; Friedrich and Beutel, 2010; Beutel et al., 2011; Ishiwata et al., 2011; Niehuis et al., 2012; Misof et al., 2014; Peters et al., 2014; Beutel et al., 2019), but the review from Trautwein et al. (2012) indicates that the ordinal status is still in question. Strepsipterans could instead be a highly specialized coleopteran.

Adult male strepsipterans have the typical characteristics associated with adult insects, such as well-developed eyes, legs, antennae, wings, and external genitalia (Kathirithamby, 1989, 2009). Adult females, however, have lost these characteristics (except for females in the basal Mengenillidae, which have legs, antennae, and eyes), and are considered neotenic, meaning they have retained their larval characteristics (Kinzelbach, 1971; Kathirithamby, 1989, 2009, 2018).

Recent taxonomic treatments indicate there are 630 described species of Strepsiptera worldwide (Kathirithamby, 2018), though this number will likely increase as more specimens are collected, and previously identified species are sequenced molecularly. Many species are likely misidentified (Straka et al., 2015) due to the cryptic nature of Strepsiptera (Kathirithamby et al., 2015). The most recent phylogeny (Fig. 1.1) designates two suborders and ten families within the order (Bahiaxenidae and Bohartillidae are not included in Fig. 1.1 as their placement within Strepsiptera is unresolved).

Members of the family Mengenillidae (suborder Mengenillidia) parasitize a basal order of insects, Zygentoma (Fig. 1.1). The females emerge from puparia, similar to the males. However, there have been observations of mengenillid females remaining inside their hosts, which may hint at how the permanently endoparasitic families of the remaining extant Strepsiptera evolved (Pohl and Beutel 2008). Males search for the free-living females, and upon finding a female, mate via traumatic insemination (Parker and Smith, 1933; Silvestri, 1940, 1941, 1943, as cited in Kathirithamby, 2018; Kathirithamby, 2009). There is a family that is considered more basal than Mengenillidae, Bahiaxenidae (Bravo et al., 2009), but due to its recent collection of only a single adult male, very little information is known about it.

The remaining extant strepsipterans belong to eight families of the suborder Stylopidia and are characterized by permanently endoparasitic females. It is debated whether or not the males of Stylopidia use traumatic insemination (Lauterbach, 1954; Beani et al., 2005; Peinert et al., 2016), or brood-canal mating (Beani et al., 2005; Kathirithamby et al., 2015) to mate with the endoparasitic neotenic female.

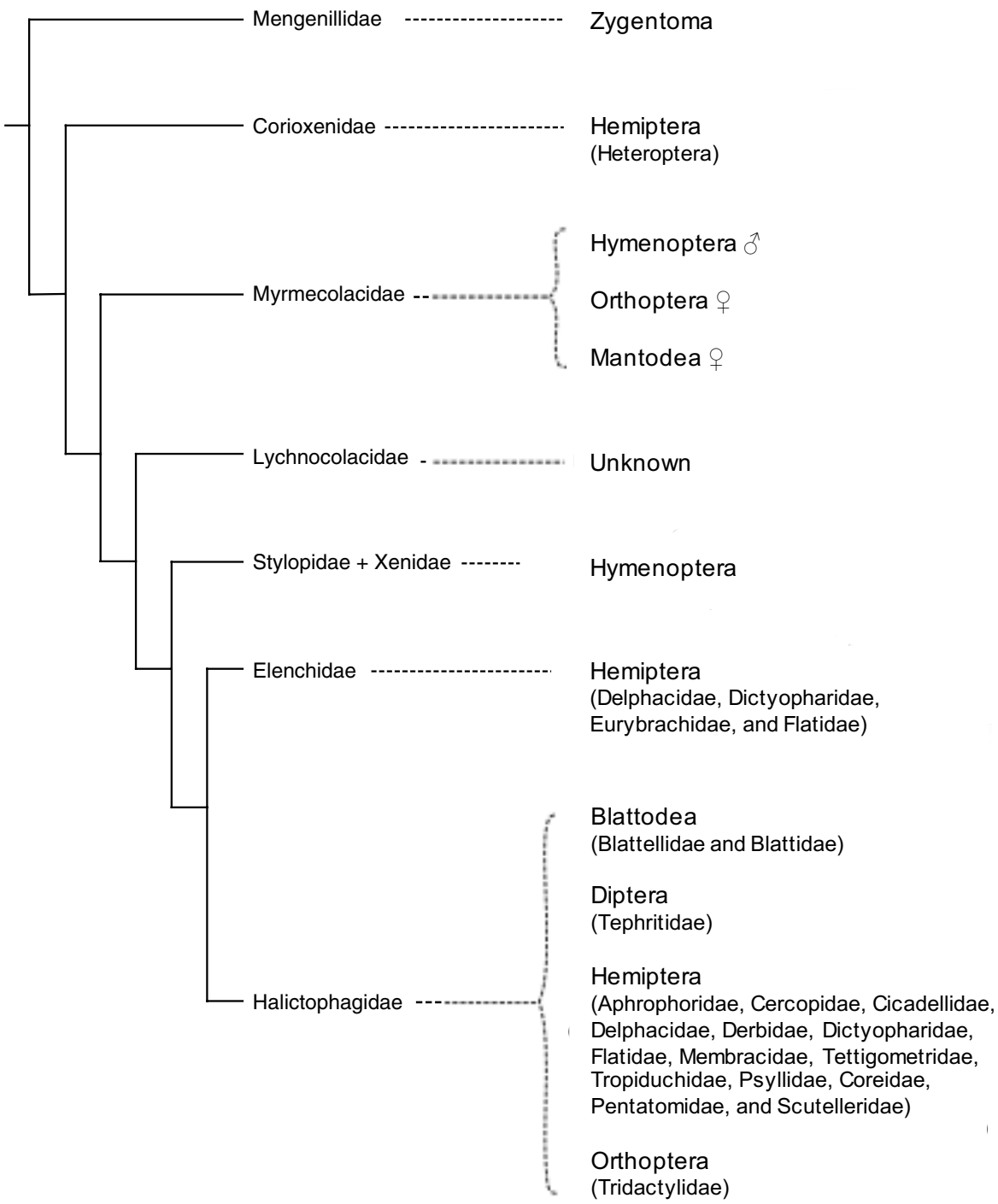


Figure 1.1. Phylogeny of Strepsiptera, with the insect order (families) that each family of Strepsiptera parasitizes, modified from Kathirithamby (2018). The strepsipteran family Bahiaxenidae (Bravo et al., 2009) is not included, but is considered less derived than Mengenillidae. Moreover, the family Bohartillidae (Kinzelbach, 1969) is not included here because its position is unresolved due to the lack of specimens collected.

Corioxenidae is the most basal of the Stylopidia and parasitizes hemipterans (Fig. 1.1). Adult males lack mandibles and instead use a ptilinum-like structure to emerge from puparia (Kathirithamby, 1989). Once a host with a strepsipteran female is located, the males use sensorial spots on their tarsi to help them detect where beneath the host's hemelytra the female is located (Pohl and Beutel, 2008; Cook and Tribull, 2013). Females of Corioxenidae do not have a brood opening like the other Stylopidia. Instead, the male inserts his aedeagus into the female's mouth opening during copulation (Pohl and Beutel, 2008).

Myrmecolacidae are an interesting family of Strepsiptera due to their host specificity (Fig. 1.1). Males of this family infect ants, whereas females infect orthopterans and mantodeans (Kathirithamby and Hamilton, 1992). Male first-instar larvae most likely require a host to take them to a nest with immature ants, whereas the female first-instars may be able to find the nymphal stages in the same area where they emerge from their mother. This method of finding a suitable host by female myrmecolacid first-instars is not confirmed, however.

The family Lychnocolacidae was originally placed within Myrmecolacidae (Kinzelbach, 1971, Kathirithamby, 1989), but upon molecular analysis was designated as a separate family, and is a sister group to Elenchidae, Halictophagidae, Stylopidae, and Xenidae (McMahon et al., 2011) (Fig. 1.1). The host of this family is unknown as only free-living males have been found (Kathirithamby, 2018).

Stylopidae is believed to be the largest family of Strepsiptera, currently with over 165 described species (Kathirithamby, 2018). However, the study by Straka et al. (2015) showed that only 67 of the 110 sequenced species of *Stylops* were valid, so Stylopidae may not in fact be the largest strepsipteran family. Stylopid parasites infect solitary bees, often *Andrena*, and the first-instar larvae require hosts to transport them to the location of a permanent host. The switch to

aculeaten hosts is considered an important step in the evolution of Strepsiptera (Pohl and Beutel, 2008). Some genera of Stylopidae (e.g., *Eurystylops*) may be parthenogenic, based on the lack of adult males collected (Bohart and Irwin, 1978), but this presumption may be due to the difficulty in collecting the adult males of these genera.

Xenidae was treated as a subfamily of Stylopidae for many years (Kathirithamby, 1989). However, molecular analysis shows that Xenidae is instead the sister group (McMahon et al., 2011) (Fig. 1.1). Members of Xenidae infect wasps, most often of *Polistes*. Similar to first-instars of Stylopidae, xenid first-instars require a host to transport them to an immature host (Hughes et al., 2003a).

McMahon et al. (2011) suggest a sister group relationship between Elenchidae and Halictophagidae. Both these families infect hemipterans, but Halictophagidae have also been found to infect blattodeans, dipterans, and orthopterans. It is suggested that a common ancestor existed that gave rise to the lineages of Stylopidae and Xenidae, and Elenchidae and Halictophagidae (McMahon et al., 2011; Kathirithamby, 2018) (Fig. 1.1).

Bohartillidae has an unknown placement within Strepsiptera due to the lack of specimens collected. Only free-living adult males have been found; thus, the host of this family is unknown. These males were placed in a new family based on the unique maxillary palp, which is covered in many microtrichia (Pohl and Beutel, 2004; Kathirithamby, 2018).

Females of Stylopidia do not pupate (Erezyilmaz et al., 2014; McMahon and Hayward, 2016), instead metamorphosing directly to their adult stage from the final larval instar. Adult males, which do pupate, can emerge from their puparia in several ways, depending on the family. The males have been observed to use a chemical solution to dissolve the cuticle along an ecdysial suture line before pushing off the cephalothecal cap (Kathirithamby et al., 1990a),

achieved using a ptilinum-like structure (Kathirithamby, 1983), or by using mandibles to cut along an ecdysial suture line (Kathirithamby, 1989; Hrabar et al., 2014).

Upon emerging from its puparium, the male is responsible for finding a female to mate with before its short adult stage ends. The difficult task of locating an endoparasitic female within her host is aided by sex pheromones released from the female's brood opening (Tolasch et al., 2012; Cvačka et al., 2012; Lagoutte et al., 2013; Hrabar et al., 2015; Zhai et al., 2016). Once a male finds a female, he will land on the host and copulation with the female parasite will commence. The sequence of copulation behaviour of *Stylops* is documented by Peinert et al. (2016). Males die soon after copulation and the females remain inside their hosts as the offspring develop. The development of the first-instars may take 30–40 days (Linsley and MacSwain, 1957), or as little as two weeks (Jones et al., 1980). The development of the embryos of *Stylops ovinae* was studied by Fraulob et al. (2015).

The neotenic females are viviparous and the mobile first-instar larvae emerge from the brood opening of the female's cephalothorax. The first-instar larva is the host-seeking stage, and targets immature stages. The strepsipteran first-instars of Corioxenidae, Elenchidae, Halictophagidae, Lychnocolacidae, Mengenillidae, and female Myrmecolacidae likely find their hosts in the same environment where they emerged from their mother. These first-instars use specialized appendages on their abdomen, called caudal filaments, to spring onto an immature host before burrowing into it (Kirkpatrick, 1937; O'Conner, 1959; Young, 1987; Kathirithamby, 1989). Male first-instars of the family Myrmecolacidae, and first-instars of Stylopidae and Xenidae infect hymenopterans, and require a host to transport them to its nest. Once they are deposited in a nest cell, the first-instars are then able to find and parasitize the host's immature

offspring (Hughes et al., 2003a; Hughes et al., 2003b; Linsley and MacSwain, 1957; Ulrich, 1956).

When first-instars find a host, they burrow into it and moult into their apodous, second-instar stage. It is likely that the first-instars use mandibles to cut the cuticle of the host to burrow within (Knauthe et al., 2016; M. Hrabar, personal communication). Kathirithamby et al. (2003) observed that when these larvae enter the host, they form a host-derived epidermal bag, which is hypothesized to protect them from the host's immune system. It is currently unclear whether the epidermal bag does protect the strepsipteran from its host's immune system, as the bag does not always completely surround the entirety of the larva's body (Manfredini et al., 2007). Observations of *Xenos vesparum* first-instars which were not completely surrounded by the host-derived epidermal bag were found to still be able to survive and develop within their host (Manfredini et al., 2007). The second-instar larvae of *Elenchus tenuicornis* were observed to undergo two subsequent moults, meaning this species has four larval instars (Kathirithamby et al., 1984). When these moults occur, the larvae do not shed their older cuticle layer; instead, it remains around them. This phenomenon has been termed 'apolysis without ecdysis', and besides presumably giving the larva protection inside its host, the function of these multiple exuvial layers is unknown (Kathirithamby et al., 1984).

Males form a puparium, using the unshed exuvia, and within it they begin to form their eyes, wings and genitalia (Kinzelbach, 1966; Buschbeck, 2005). The females extrude their cephalothorax from an abdominal tergite or sternite, and the cephalothorax soon undergoes sclerotization (Cook, 2014). The life cycle continues as the males emerge and begin looking for a female.

1.2 Biology of *Stylops*

The strepsipteran family Stylopidae is considered the largest family of Strepsiptera (Kathirithamby, 2018), but the number of species within this family is likely incorrect, as shown by Straka et al. (2015). *Stylops* is the second largest genus in the Strepsiptera with around 67 described species (Straka et al., 2015), behind *Halictophagus* (Halictophagidae) (Kathirithamby, 2018). Stylopidae is a cosmopolitan family, made up of nine genera which include *Halictoxenos* and *Crawfordia*. Bohart (1941) noted 48 species of Stylopidae could be found in North America. A recent study by Straka (2019) found 15 species of Stylopidae in Canada, but the number of which were *Stylops* was not included. *Stylops* parasitizes many species of an important bee pollinator, *Andrena*. Many species of *Stylops* are strict specialists, only infecting one species of *Andrena*, though some *Stylops* species infect several and are considered generalists (Jůzova et al., 2015).

For my master's research, I studied *Stylops advarians*. Specimens of *S. advarians* were found to infect *Andrena milwaukeensis* in Saskatoon, Saskatchewan. *Stylops advarians* is a specialist of the subgenus *Andrena*, as it has been found parasitizing three other *Andrena* species: *A. frigida*, *A. mandibularis*, and *A. vicinoides* (Straka et al., 2015). The life cycle of *Stylops* is similar to that detailed in Section 1.1, but a unique way that the first-instar larvae travel with an adult bee to find an immature bee has been observed. Linsley and MacSwain (1957) found live first-instar larvae of *Stylops pacifica* inside the crop of several non-stylopized bees. The same authors suggested that these larvae were getting ingested by the non-stylopized bees as they foraged for nectar from *Ranunculus* flowers. These authors go on to suggest that the non-stylopized bee inadvertently transports the first-instar larva back to its nest where it will then regurgitate the nectar, along with the first-instar, into a nest cell. Once the bee has deposited

enough food for its offspring, it will deposit an egg and seal the nest cell, where a first-instar resides. A first-instar larva of *S. pacifica* was observed penetrating an egg of *Panurginus melanocephalus* (Linsley and MacSwain, 1957). Although *P. melanocephalus* is not the typical host of *S. pacifica*, it suggests that *Stylops* first-instars, unlike other strepsipterans, initially infect the egg of their host, rather than the larval or nymphal stage. The findings of Knauthe et al. (2016) also support the hypothesis that *Stylops* first-instars target the egg instead of the larval stage, based on the morphology of the head of *S. ovinae*.

Stylops advarians was first described from a single adult female by Pierce (1909), who included a drawing of the cephalothorax. Bohart (1941) included his own drawing of the female's cephalothorax, but did not mention any collection of the adult male. The male was thought to be the only stage that could be used to accurately describe a species, due to the lack of identifiable characteristics of the adult female. However, it has recently been found that using a male to identify the species is still difficult, and not accurate enough. Kathirithamby et al. (2015) indicate that Strepsiptera is a cryptic group, so even the males cannot be accurately distinguished from one another. To correctly identify our species of Strepsiptera, several first-instars underwent DNA sequencing (see Section 2.3.3) with the help of the Chilton Lab at the University of Saskatchewan. The sequences were found to be a 99% match to the sequence of *Stylops advarians* uploaded by Jůzova et al. (2015) to the Basic Local Alignment Search Tool (BLAST, blast.ncbi.nlm.nih.gov/Blast/cgi).

1.3 Objectives and Chapter Synopses

The objectives of our study were to examine the structure of three life stages of *Stylops advarians*, critical to understanding the life history of this parasitic species, as well as to examine the impacts that these parasites have on their host.

Chapter 2 covers the ecological and life history aspects of *S. advarians* that I examined. The prevalence, intensity and mean intensity, and abundance of these parasites within a population of *Andrena milwaukeensis* near the South Saskatchewan River in Saskatoon, Saskatchewan, were recorded. Only bees with adult females were collected consistently, so our data reflects the number of female parasites in the bee population, but not the number of bees affected by males. Over consecutive field seasons, I kept records of where and when stylopized bees were first observed, where these stylopized bees were found over their foraging season, and when the first-instars of *S. advarians* began to emerge.

Chapter 3 covers the morphology and anatomy of three life stages of *Stylops advarians*: the adult male, adult female and first-instar larva, as well as the impact the adult female has on the host's anatomy. The adult male of *S. advarians* was collected for the first time, and is described. Next, the structure of the adult female was examined, which mainly focused on the morphology of the cephalothorax. Finally, I wanted to examine the structure of the first-instar larvae and compare it to the first-instars of *Stylops* that have already been described (Pohl, 2000; Knauthe et al., 2016). This study marks the first morphological investigation of a non-European species of *Stylops* and one of the few investigations on Canadian Strepsiptera.

As the concluding chapter, Chapter 4 sums up all of our findings as well as provides directions that further research could take on *S. advarians* and other strepsipteran species.

1.4 Thesis Format

Parts of the research results of this thesis were written as manuscripts for publication in the format befitting a particular research journal. Thus, the reader will encounter some differences in writing style within the thesis, and may find repetition of some information throughout the text.

CHAPTER 2: HOST-PARASITE INTERACTIONS BETWEEN *STYLOPS ADVARIANS* AND *ANDRENA MILWAUKEENSIS*

2.1 Abstract

Specimens of *Stylops advarians* were collected by acquiring their host, *Andrena milwaukeensis*, near Cosmopolitan Park in Saskatoon, Saskatchewan (52°07'43.8"N, 106°38'50.2"W–52°07'18.0"N, 106°39'25.2"W). Overwintered adult bees of *A. milwaukeensis* were initially found foraging on *Shepherdia* in the northwest area of Cosmopolitan Park in early May. As the season progressed, bees were found increasingly southwest, foraging on *Syringa* and *Cotoneaster*. Each year from 2016–2018, bees were active beginning around May 2 and ending around June 22; therefore, bee collection occurred regularly during this 7-week period. Most bees (over 75% each year) were not infected by *S. advarians*, and those that were typically had just one parasite. Occasionally, a bee with two parasites was collected, but only rarely were bees detected with three. Only female *S. advarians* were collected consistently, and were most often found with the cephalothorax protruding from the host's abdomen between the 4th and 5th tergites, equally on the left or right side of the bee. Over the three years of sampling, the prevalence of stylopized bees remained around 22% each year, higher than has been reported in other studied species of Strepsiptera that parasitize Hymenoptera. Intensity was 1–3 female parasites per bee, with a mean of 1.2. Parasite abundance was 0.27. A high proportion of the female bees initially captured each year (i.e., May 2 and soon after), were stylopized. However, non-stylopized female bees typically were not encountered until around ten days later. First-instar larvae of *S. advarians* were first observed from May 22 – 25 each year, which suggests that adults of *S. advarians* matured and mated at similar times during these three consecutive years.

2.2 Introduction

There is little data on the prevalence of hosts infected with Strepsiptera. The studies that are available have focused on Stylopidae and Xenidae, whereas the other strepsipteran families (see Fig.1.1) have not been studied in this regard. Hughes et al. (2004) found that 7% of *Polistes dominulus* collected from nests were infected by *Xenos*, but did not find many stylopized wasps away from the nest. However, it was later found that 25% of overwintering female wasps were infected (Hughes et al., 2004). Pierce (1909) provides prevalence data for *P. annularis*, and found that 17.2% of wasps, from two separate nests, were stylopized. Jones and Jones (1981) examined the prevalence of *Stylops crawfordi* females by collecting *Andrena crawfordi*, and found it initially to be above 25% before dropping to under 10% a few days later. Of the stylopized bees examined, very few had a male, determined by the presence of a puparium. When a puparium was observed, it was always empty (Jones and Jones, 1981).

The difficulty of finding males could be due to their timing of emergence. Hrabar et al. (2014) found that females and males of *Xenos peckii* have a synchronous maturation, meaning males emerge from their puparia at the same time that females protrude from their host's abdomen. This synchronicity increases the chances of males finding females. A behavioural change has been observed in female *Andrena* infected by *Stylops* (Linsley and MacSwain, 1957; Straka et al., 2011). Male *Andrena* normally emerge earlier than females (Westrich, 1989, as cited by Straka et al., 2011), but stylopized females were observed emerging at the same time as non-stylopized, and stylopized, males (Linsley and MacSwain, 1957; Straka et al., 2011). This early emergence of stylopized female bees would enable all male and female *Stylops* to be outside of their host's nest, increasing the chances that once they are mature they can find a mate. The males of some *Stylops* species have been observed mating with a female while her

host bee is foraging (Linsley and MacSwain, 1957), whereas others were only found mating with females at the bee's nest site (Ulrich, 1933). If male *Stylops* are not collected while in their puparia, or the nest site of the host bee is unknown, it can be extremely difficult to find these short-lived males of *Stylops*.

The first objective of this research chapter of my thesis was to determine the species identity of my *Stylops* specimens using molecular methodology. Next, I recorded several life history traits, such as the seasonal dates of the parasite's activity, including initial emergence of the stylopized hosts (*Andrena milwaukeensis*) and the emergence of the host-seeking *Stylops* larvae from the adult female parasites, the prevalence, intensity, and abundance of this parasitic species within its host's population near central Saskatchewan, and the predominant location of the protruding, mature parasites within the adult host's body.

2.3 Methods

2.3.1 *Study Site*

Adult females of *Stylops advarians* were collected by capturing their foraging host, bees of *Andrena milwaukeensis*, from 2016 to 2018. These bees could be found in Cosmopolitan Park in Saskatoon, SK, near the South Saskatchewan River. Each spring, bees of *A. milwaukeensis* were found foraging on *Shepherdia* flowers at the northwest region of the site (52°07'43.8"N, 106°38'50.2"W) in early May, and then 1.1 km southwest (52°07'18.0"N, 106°39'25.2"W) several days later on *Cotoneaster* and *Syringa*, as the season progressed.

2.3.2 *Specimen Collection and Analysis for Parasitism*

Collection began in 2016 as part of my honour's project, and continued in 2017 and 2018 for my graduate research. Adult bees of *A. milwaukeensis* were collected by sweep netting and placed in vials. When foraging, these bees could be quickly identified due to their red/orange tergal hairs. These bees can be more accurately identified as *A. milwaukeensis* by their long clypeus, and rounded pygidial plate (LaBerge, 1980) Back at the lab, these bees were examined for the presence of strepsipteran parasites using an Olympus SZ-ST dissecting microscope. Other methods of collection were also deployed, such as malaise traps and nest emergence traps, but these were unsuccessful. The non-stylopized bees, and most of the stylopized bees, were euthanized using vapours from ethyl acetate, then pinned, labelled, and stored inside a Schmitt Box. Stylopized bees that were not immediately euthanized were placed into cloth mesh bags to attempt to lure adult males of *S. advarians* to their location, or to collect first-instar larvae that emerged from the protruding female parasites in late May.

2.3.3 *Species Identification*

Total genomic DNA was extracted from the entire bodies of 4–6 first-instar larvae (pooled together) from a single mother, as well as from the entire adult male found inside a puparium within the same host as the male that was described morphologically (outlined later, in sections 3.3.1.1 and 3.4.1). Extraction was done according to the manufacturer's protocol of DNeasy Blood and Tissue Kit (Qiagen), and stored at –20 ºC. Amplification of 710 bp target regions of cytochrome oxidase I was done using Polymerase Chain Reaction (PCR) conditions described in Folmer et al. (1994). PCR contamination was checked through gel electrophoresis analysis. Amplicons were purified using exonuclease I – shrimp alkaline phosphatase

(Krakowetz et al., 2015) subjected to automated DNA sequencing using primers (LCO1 - 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198 – 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') in individual reactions. The DNA sequences of our specimens were compared to the sequences of other *Stylops* species found in the GenBank database of the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/genbank/) using Geneious x.x.x (https://www.geneious.com)

2.4 Results

2.4.1 *Identification as* Stylops advarians

Based on the sequencing data acquired from the first-instar larvae (see Section 2.3.3), it was concluded that our specimens belong to *Stylops advarians*. Two base pairs (156 and 319) were found to be mismatches from the *S. advarians* sequence found by Jůzova et al. (2015), yielding a 99% match (www.ncbi.nlm.nih.gov/genbank). The *S. advarians* specimens sequenced by Jůzova et al. (2015) were removed from *A. milwaukeensis* taken at Cornwall, New York and Hancock, Maine. The sequencing results of the adult male absolutely matched that sequence acquired from the first-instar larvae. Therefore, we are confident that our specimens from this study population belong to *S. advarians*, despite the larvae being sampled in 2017 and the adult male being collected the following spring.

2.4.2 *Seasonal Occurrence of* Stylops advarians

In 2017 and 2018, adult females of *Stylops advarians* were encountered as early as May 2 around the northeastern area of the study site (Fig. 2.1, points 1 and 2). Overall, only adult females of *S. advarians* were found upon inspection of their host, *Andrena milwaukeensis*, with

the exception of one bee collected on May 14, 2018 that had two intact puparia (males). Only female bees were collected in 2016 and 2017. Male bees were caught in 2018, but were not observed after May 18 (Appendix). Male bees were also found to be stylopized. Bees of *Andrena milwaukeensis* were no longer found at this location after May 8, but instead were found southwest of the initial foraging location 6–10 days later (Fig. 2.1, point 3), where they visited flowers of *Cotoneaster* and *Syringa*. Both stylopized and non-stylopized bees were collected further southwest along the Meewasin Trail as the season progressed (Fig. 2.1, points 4–6), until around June 22. After June 22, *A. milwaukeensis* could not be found. By moving further southwest I was able to continually collect *A. milwaukeensis*, and thus collected *S. advarians* specimens throughout May and June of each year.

From 2016–2018, the intensity of parasitism was 1–3 adult females of *S. advarians* per bee. The appendix lists dates of collection, sex of the collected bee, presence of stylopization, and numbers of *S. advarians* per bee. However, our frequency data summarized here does not differentiate between bees that had one, two, or three female *Stylops*. I only considered if they had zero *Stylops* or at least one. The frequency of stylopized bees collected per day did not show a clear pattern over the three-year sampling period (Figs. 2.2–2.4), except for early in the season. Stylopized female bees were regularly caught around May 2, with numerous days early in each spring where all bees collected were stylopized (Figs. 2.3, 2.4). Non-stylopized bees were rarely seen from May 2 – 4 (Figs. 2.3, 2.4). In 2016, collection did not take place at the northeast region of the study site on May 2, so I am unable to determine if stylopized bees were present in this area at that time (Fig. 2.2).

No seasonal pattern for the occurrence of multiple *S. advarians* per collected host was apparent. Instead, such hosts were encountered randomly. For example, in 2017, the four bees

with two *S. advarians* in their gaster were taken on May 5, 15, 29, and June 12. Corresponding collection dates for eight similar hosts in 2018 were May 2, 4, 22, 28, June 4, 6, 12, and 18 (see Appendix).

First-instar larvae were observed emerging from the brood opening of several endoparasitic females at similar times each year. In 2016, first-instars were initially observed on May 25. In 2017, the host-seeking larvae were first observed on May 24, and in 2018, first-instars were observed on May 22. Larvae were found to have asynchronous development inside their mother's body (Kathirithamby, 2009; see Section 3.4.2.2). Thus, many more larvae were still found emerging at later dates, as well as from within the same female's body, after the initial observed emergences each year.

In 2019, some additional stylopized bees were collected strictly for anatomical investigations. Surprisingly, *A. milwaukeensis* was found two weeks earlier than expected, but at a different location. Near the parking lot of the Collaborative Sciences Research Building (CSRB) at the University of Saskatchewan (52°07'56.4"N 106°37'57.6"W), several stylopized bees were collected on April 17. Only stylopized female bees were observed, similar to our observations in 2017 and 2018, as well as the observations of Straka et al. (2011).

2.4.3 *Prevalence, Intensity, and Abundance*

Bees were collected from 2016 to 2018, inclusive, and the number stylopized were noted (Table 2.1; Appendix). These results only include the bees that had females within, due to the very few bees collected that had intact or empty puparia (see below).

The maximum number of *Stylops* females found in one bee abdomen during the collection period was three. Of the total of 455 bees of *A. milwaukeensis* that were collected

Table 2.1. Number of bees collected each year, including the number of *Stylops* females found in each host. Prevalence, mean intensity, and abundance for each year of collection are indicated in the rightmost columns.

Year	Number of female *Stylops* per bee				Total bees collected	Total bees stylopized	Prevalence	Mean Intensity	Abundance
	0	1	2	3					
2016	97	27	1	2	127	30	24%	1.17	0.28
2017	102	23	4	1	130	28	22%	1.21	0.26
2018	157	32	8	1	198	41	21%	1.24	0.26

during May and June of 2016–2018, 356 (78%) did not have a female parasite (Fig. 2.5). Eighty-two bees (18%) had one female parasite (Figs. 2.5, 2.6A). Thirteen bees (3%) had two female parasites (Figs. 2.5, 2.6B), and 4 bees (1%) had three female parasites (Figs. 2.5, 2.6C). From Table 2.1, the mean intensity of parasitism was 1.2 and the mean abundance was 0.27. Thus, most bees collected did not have any sign of parasitism by a female *S. advarians*.

Most adult females of *S. advarians* were found to protrude from between the 4th and 5th tergites on the left or right side of the host bee's metasoma. No significant difference was found regarding which side of the host's abdomen the female parasite occupied ($X^2_{1df} = 1.634$; N = 42 bees, p=0.4389) In cases where three females were present, a female could be found occupying the left and right side of the host, and the third could be found in between these two, in the centre of the bee's abdomen (Fig. 2.6C). Rarely was a bee found that had a single female residing in the centre of its abdomen (Fig. 2.6D). Linsley and MacSwain (1957) found that these individual females in the centre often had undeveloped *Stylops* on either side of them. I was unable to find similar results to their findings when examining the few collected bees that had one central female. When a bee infected with one central female parasite was found, that parasite was wider than those that were found laterally in a bee's abdomen.

Two abnormal specimens were acquired in 2019 during collection for anatomical comparison between stylopized and non-stylopized bees. One bee collected on April 17 at the CSRB location (see Section 2.4.2) had evidence of four *Stylops*: two puparia (one empty, one intact) and two females (Fig. 2.6E). The empty puparium and two females protruded from between the 4th and 5th tergites, but the intact puparium protruded from between the 3rd and 4th. Another host specimen collected on April 17 was found with an empty puparium on the ventral

side of its body, protruding from between two sternites (Fig. 2.6F). Both host bees were captured while foraging on *Salix* catkins.

2.5 Discussion

Our results regarding the timing of emergence of stylopized female bees are similar to the observations of Linsley and MacSwain (1957) and Straka et al. (2011). As suggested by Straka et al. (2011), *Stylops* females may manipulate their bee host, if that overwintered host is female, to emerge from her nest earlier than normal. If the host is male, then no manipulation is required as the males normally emerge earlier than females (Westrich, 1989, as cited by Straka et al., 2011). A consequence of this manipulation is that stylopized female bees emerge at the same time that stylopized male bees do, thereby increasing the amount of strepsipterans throughout the environment; as a result, the chances of each male finding a female would increase.

Free-living adult males were difficult to encounter over the three years of sampling. This difficulty was likely due to the synchronization of the emergence of males from their puparia with female maturation (Hrabar et al., 2014), and their short life span (Kirkpatrick, 1937; Kathirithamby, 2009). Linsley and MacSwain (1957) observed *S. pacifica* males mating with females as the female's host bee was foraging at *Ranunculus californicus* flowers. Stylopized bees of another *Andrena* species were observed to emerge earlier than normal, but did not leave their nest site, presumably because flowers had not begun blooming yet (Ulrich, 1933). I suggest that stylopized *A. milwaukeensis* near Cosmopolitan Park emerge before the flowers they feed on bloom; thus, mating of *S. advarians* likely occurs at the bee's nest site. Unfortunately, nest sites of *A. milwaukeensis* were not found during my study, so this supposition has yet to be confirmed.

The mating period of *S. advarians* likely occurs on, or shortly before, May 2, when

stylopized bees were first observed. This conclusion is based on factors such as when stylopized bees were first found each spring, the synchronization of male emergence and female maturation (Hrabar et al., 2014), and the lack of intact males within bee hosts collected after May 2. The acquisition of a bee with two intact puparia collected on May 14, 2018 is likely because these two males were unable to emerge, rather than because mating of *Stylops* occurs over a two-week period. The two males were found deceased within their puparia, likely due to their short life spans (Kathirithamby 2009), after the bright light of a microscope lamp was shone on them to attempt to coax them to emerge (see Section 3.3.1.1). The study by Jones and Jones (1981) also suggests that all males emerge and mate around the same time, as they likewise could only find empty puparia.

Only female parasites were consistently collected due to the extremely short time that adult males were active. Since females reside within their host as adults, I collected *A. milwaukeensis* bees throughout their foraging season to collect these female parasites. Stylopized bees were observed on the early blooming flowers of *Shepherdia*, and non-stylopized bees were much less commonly seen at this time. Stylopized and non-stylopized bees were later found further southwest on *Syringa* and *Cotoneaster*. The bees' movements were likely due to the depletion of the pollen and nectar sources from each bush these bees fed on. Once floral resources of pollen and nectar had been exhausted owing to pollination and seed/fruit set at each location, the bees moved to the next area that had newly-opening flowers.

First-instar larvae of *S. advarians* were observed to first emerge at similar times each year. This comparable emergence in three consecutive springs suggests that mating between males and females occurs at similar times each year, assuming the length of development from embryo to first-instar remains consistent year-to-year. Linsley and MacSwain (1957) suggest that

the development time of *S. pacifica* first-instars, from insemination to emergence, is 30–40 days. Jones et al. (1980) found the incubation period of the eggs of *S. bipunctatae* to be 2–3 weeks. The finding of eggs within adult females around May 2 (see Section 3.4.2.2), as well as the emergence of first-instar larvae around May 22, suggests that the duration of the incubation period of *S. advarians* resembles that of *S. bipunctatae*.

Within stylopized hosts, only females of *S. advarians* were collected consistently. Thus, the prevalence data only consider the number of bees stylopized by at least one female, with the collected bee having two puparia (males) not being included. The prevalence is undoubtedly higher than 22%, but I was unable to determine the number of bees infected by male parasites. Further research is required to determine the sex ratio of the first-instar larvae, which may clarify the true prevalence of *S. advarians* within this population of *A. milwaukeensis*, assuming both sexes have the same chance of successfully parasitizing a host.

The prevalence of stylopized bees remained consistent (21–24%) over the three consecutive years of sampling. This consistency may be due to the vast numbers of larvae that each female produces, as well as the minute chance that each first-instar has of successfully parasitizing a permanent host. Since so many first-instars are produced by each female, there may not be much fluctuation in the quantity of these larvae that successfully find a host from year-to-year. This presumably low success rate would cause the number of bees that become infected with *Stylops* to remain similar each year, leading to similar prevalences.

The intensity of parasitism throughout the collection period of 2016–2018 was never more than three females per host. Most of the 455 bees (78%) were not stylopized, and the majority of those that were had only one parasite (18%). Occasionally, bees were found with two female parasites (3%), but very rarely (1%) were three female parasites present in a host. The

mean abundance of parasitism was 0.27, and the low mean intensity (1.2) of *S. advarians* in *A. milwaukeensis* bees is most likely due to the difficulty of a first-instar successfully finding and surviving within a permanent host. First-instars are deposited on flowers and picked up by an adult bee host, which brings the first-instar to its nest (Linsley and MacSwain, 1957; and see Section 3.4.4.1). The larva on the bee is inadvertently deposited into a nest cell of the adult bee host where it can then burrow into a permanent immature bee host (see Section 3.2). It is likely rare for a single larva on a flower to successfully travel with an adult bee host to its nest, via phoresy, and parasitize one of that host's offspring. It is therefore a rarer event for two or three strepsipteran first-instars to be picked up by a single adult bee host, travel to the bee's nest, and then successfully parasitize the same immature bee in the host's nest.

Females were most often found protruding between the 4th and 5th tergites of their host's abdomens, even when three females were present. The consistent location of the females within their host is likely because this position provides the female with the most reproductive success (Maeta et al., 2001). This position allows the female to grow to the anterior end of her host's gaster, but also not impact important organs of the host in a debilitating way. A longer body may allow the female to produce more offspring. Female parasites were also often found in the lateral area of their host's abdomen in single- and double-parasite infections.

Occasionally, some bees were found to have only one female *Stylops* protruding from the midline of their gaster and, unlike the findings of Linsley and MacSwain (1957), I did not find undeveloped *Stylops* on either side of this single central female. The few central females I found were wider than those found laterally, and perhaps this larger space permits increased growth of the parasite that allows more larvae to be produced. However, if the number of eggs a single female produces is genetically based, as observed in *Drosophila* (Robertson, 1957) or is

governed by nutrient acquisition (Rivero et al., 2001), the lateral or central placement within the host would be irrelevant in regard to larva production. The observation of most females found laterally likely has to do with how the female's body is supported by her host's organs (see Section 3.4.3).

The prevalence of a few other strepsipteran species is known. Some of these studies were able to determine the number of hosts infected with both males and females (see Hughes et al., 2004). Jones and Jones (1981) initially found similar prevalences to my results with female *S crawfordi* infecting an *A. crawfordi* population, but prevalence soon dropped to under 10% for the remainder of their collection period. The latter lasted around 30 days, but collection only occurred on 13 of these days due to rain (Jones and Jones, 1981). Their results are rather incomplete as they were unable to sample consistently throughout the foraging season of *A. crawfordi*.

Prevalence may be different between strepsipteran species due to several factors. The environment that these parasites and their hosts live in may play a large role in how successful each parasite is. The behaviour of the parasite and host can also impact the prevalence. For example, the behaviour of stylopized *Polistes* is different than that of stylopized *Andrena* hosts. Stylopized *Polistes* wasps forego their social tasks and leave their colony to gather at the same site, enabling the parasite to mate (Beani et al., 2011). *Andrena* infected by *Stylops,* however, continue their normal behaviour of foraging and nest making (Linsley and MacSwain, 1957). Strepsipterans may also manipulate their host, as seen with *Xenos* females potentially causing their wasp host to spend more time on *Campsis radicans* flowers in order to increase the success of their larvae being deposited in the proper place (Beani et al., 2018). The prevalence of most other strepsipteran families is unclear and needs further study.

The prevalence, intensity, and abundance from 2016–2018 is consistent and suggests that the host-parasite system of *S. advarians* and *A. milwaukeensis* is currently in balance. It is unknown what contributes to this consistency, but it may be due to several factors, including the number of larvae produced by the female parasites, the number of these larvae that are successfully deposited onto flowers to await an adult host bee on which these larvae can travel, the chances of multiple larvae being deposited on the same flower, the success of first-instars attaching to, or being ingested by, an adult bee host, the foraging behaviour of the adult host bees, and the developmental success while inside a host.

In 2019, several specimens of *A. milwaukeensis* were collected near the CSR Building on April 17, as they foraged on *Salix* catkins. This date is about two weeks earlier than bees of *A. milwaukeensis* were normally collected during 2016–2018. On April 17, 2019, I examined my study site and found no bees active. I suggest that the bees found near the CSRB have nests near the building, and that the ground warmed more quickly than the ground at my study site of 2016–2018, perhaps due to the recent construction or presence of the building. The temperature increase may have initiated the emergence of the bees found at the CSRB, as reported elsewhere (Schwartz and Karl, 1990; Schenk et al., 2018). One of these bees was infected with four *Stylops* parasites. The presence of four *Stylops* in one bee of *A. milwaukeensis* is likely an extremely rare event, as this is the only specimen with four *Stylops* found in over three years of sampling. Another of these collected bees had an empty puparium on the ventral side of its abdomen. I suggest that this strategy could work for males, as the male was able to emerge from this position and successfully fly away, but it would be detrimental for females as the male would have to land on the host's ventral side to mate with the female. Protruding from the tergites rather than the sternites is still likely the most successful strategy, as well as the most often seen. As far as I

know, this recent finding is the first recorded instance of a *Stylops* specimen being found to emerge on the ventral side of its host's abdomen. However, it is a common observation in *Polistes* stylopized by *Xenos* (Beani, 2006).

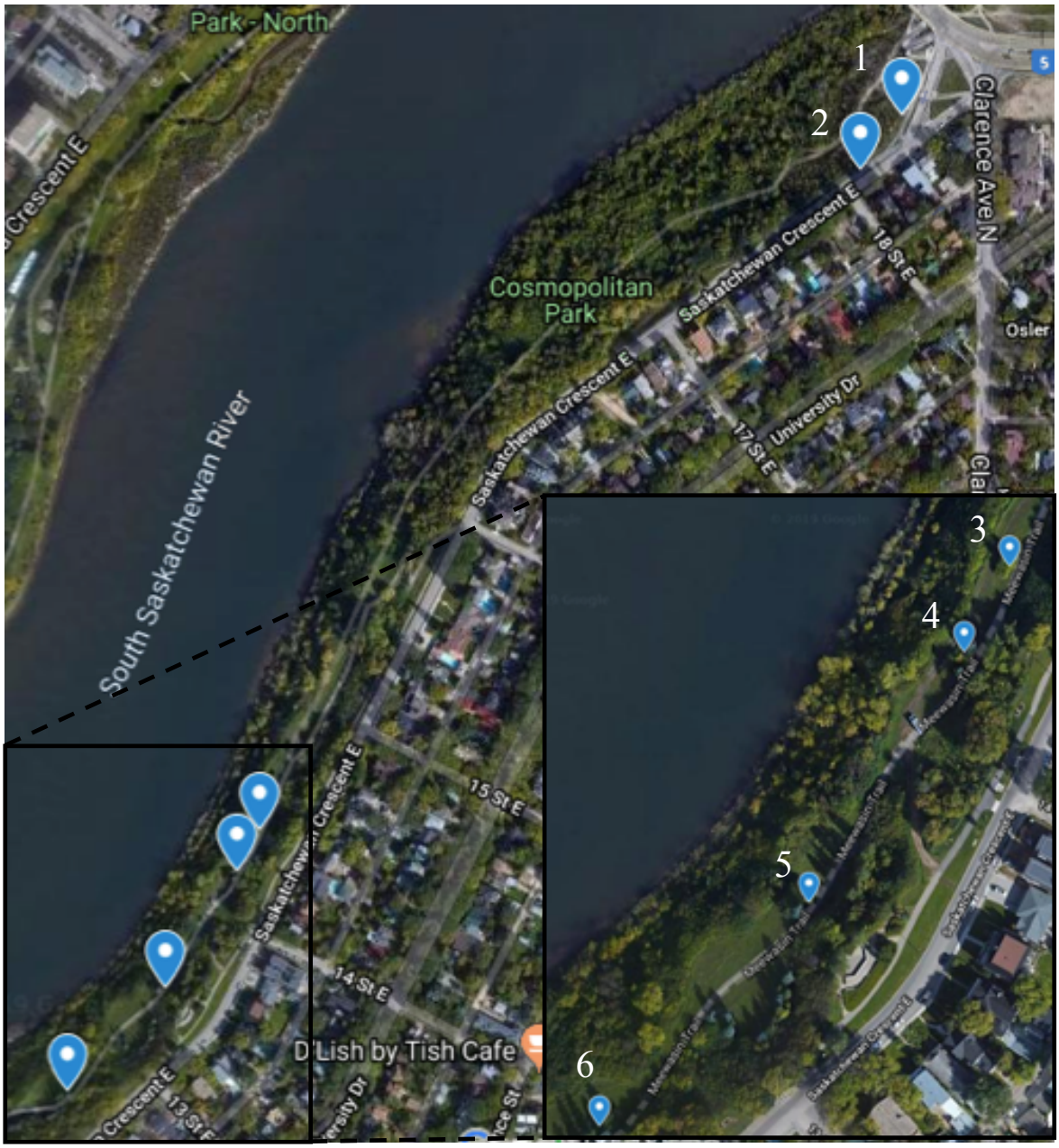


Figure 2.1. Map of the study site where foraging bees of *Andrena milwaukeensis* were collected to sample *Stylops advarians*. Points 1 and 2 show where the bees could be found in early May of each year in 2016–2018. Points 3–6 depict where the bees were found later in the season. As the season progressed, the bees were found southwest at point 3, then point 4, point 5, and finally at point 6. The inset shows the area of points 3–6 in greater detail. The bees were not found after June 22 at point 6, nor anywhere else near the study site. Images are courtesy of Google Maps.

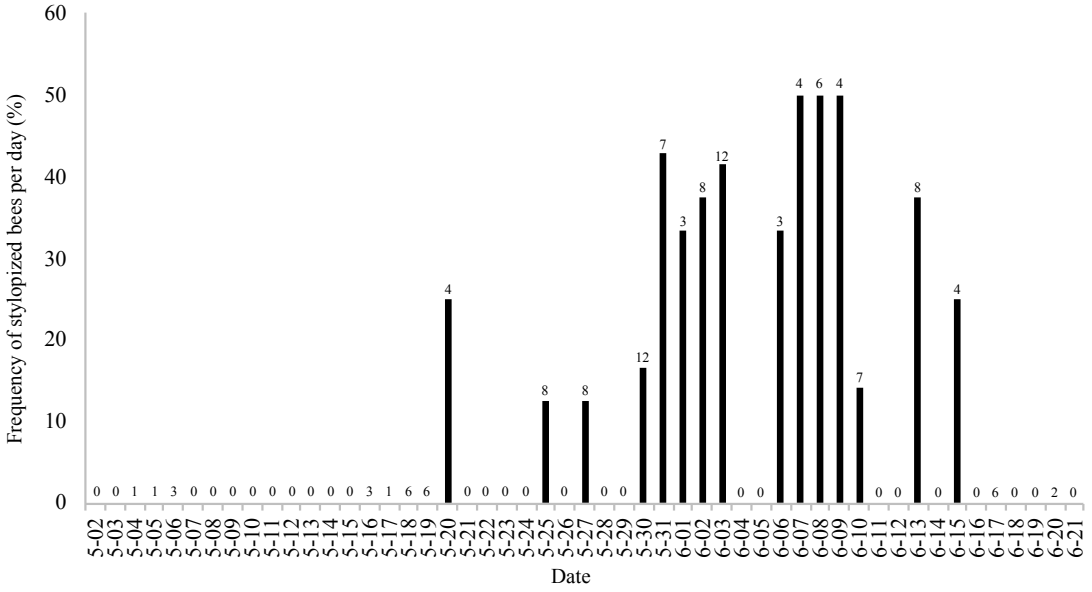


Figure 2.2. A bar graph showing the frequency of stylopized bees of *Andrena milwaukeensis* collected each day in 2016, as part of my honour's project. The number above each bar is the total bees collected during that day. Dates without bars are either days where collection did not occur, or no stylopized bees could be found.

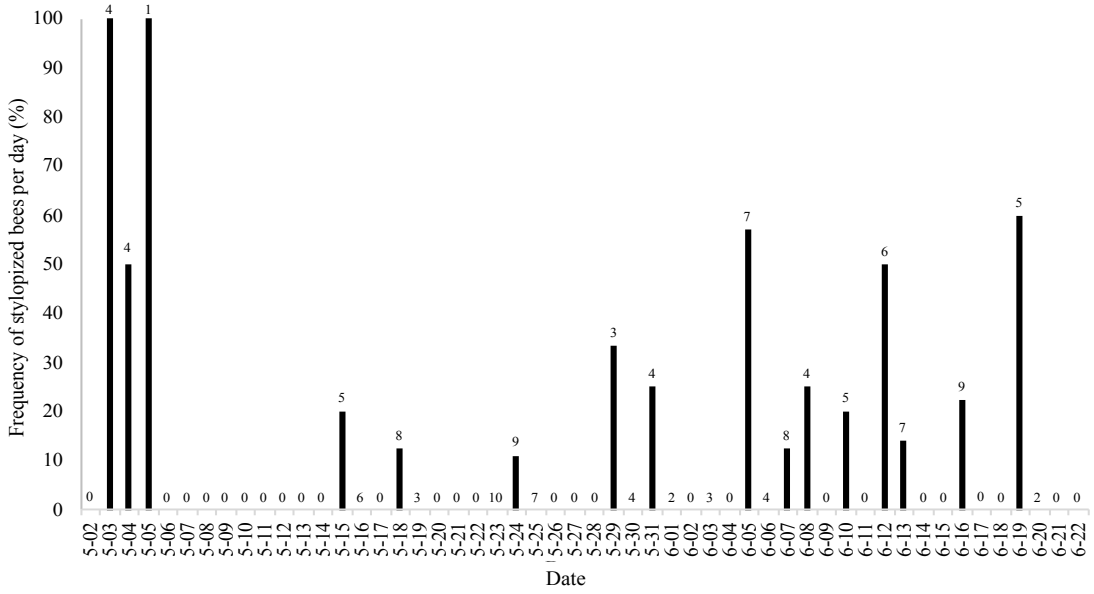


Figure 2.3. A bar graph showing the frequency of stylopized bees of *Andrena milwaukeensis* collected each day in 2017. The number above each bar is the total bees collected during that day. Dates without bars are either days where collection did not occur, or no stylopized bees could be found.

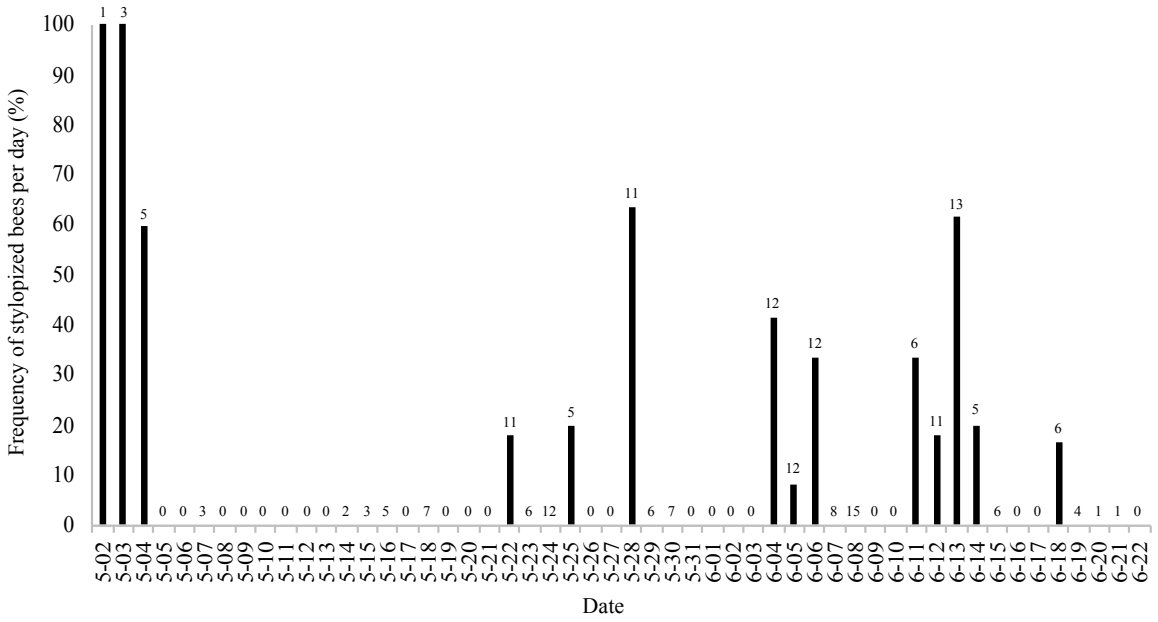


Figure 2.4. A bar graph showing the frequency of stylopized bees of *Andrena milwaukeensis* collected each day in 2018. The number above each bar is the total bees collected during that day. Dates without bars are either days where collection did not occur, or no stylopized bees could be found.

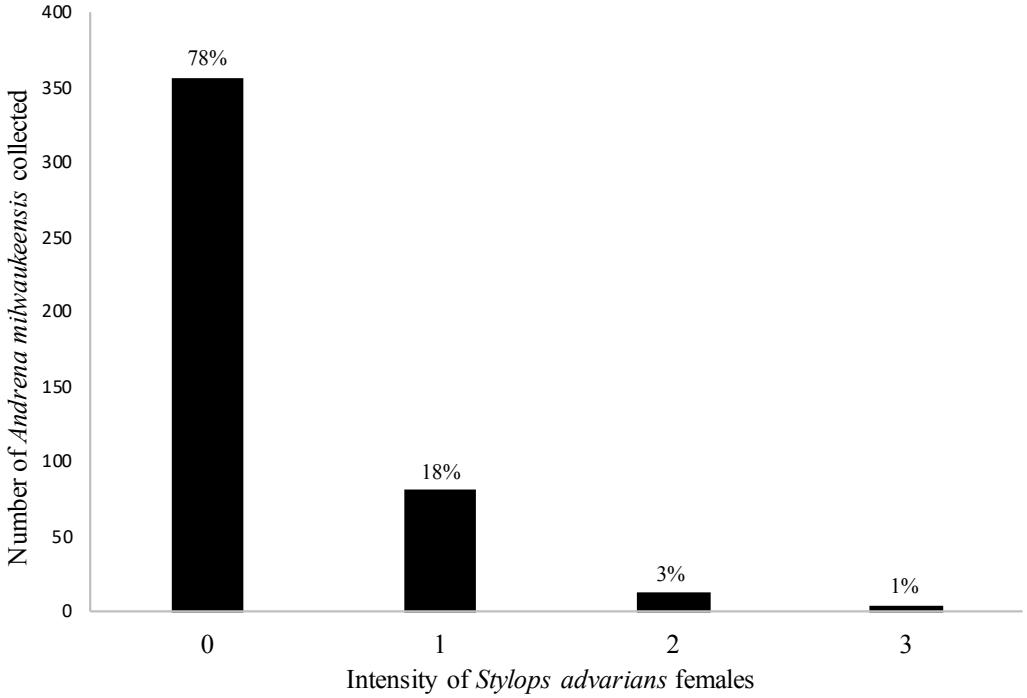


Figure 2.5. A bar graph showing the intensity of infection by adult females of *Stylops advarians* within the *Andrena milwaukeensis* population in Saskatoon, SK sampled from 2016–2018. Percentages are shown above each bar.

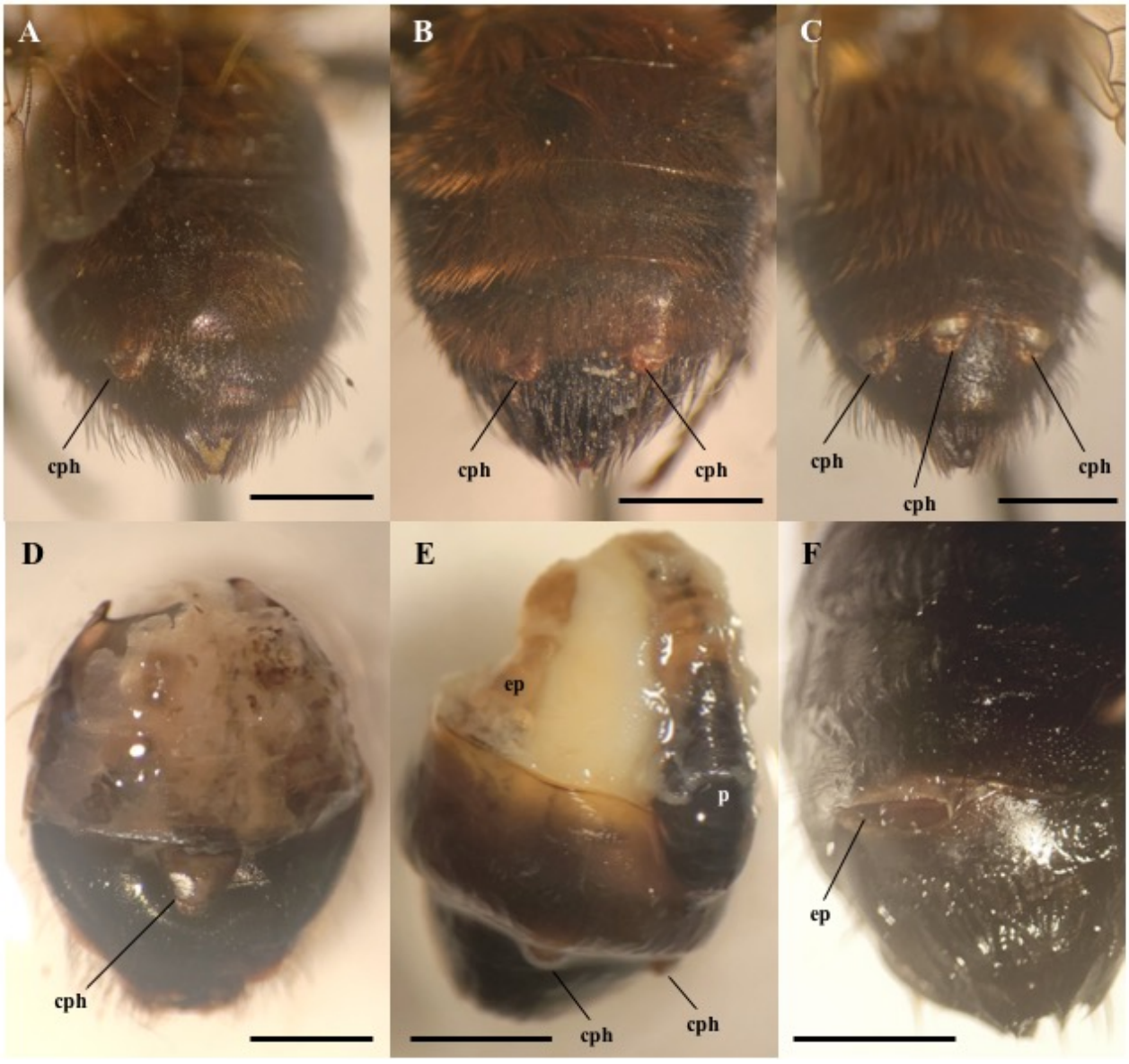


Figure 2.6. Photographs of dorsal (A-E) and ventral (F) surface of abdomens of *Andrena milwaukeensis* infected with *Stylops advarians*. **A**: Bee abdomen stylopized by one adult female. Note protruding cephalothorax (cph) of the adult female parasite. **B**: Abdomen stylopized by two adult females. **C**: Abdomen stylopized by three adult females. **D**: An adult female at the midline of the bee abdomen. **E**: Partially dissected bee abdomen with two females (cph) and evidence of two males (ep, p). **F**: Abdomen of bee found alive, showing an empty *Stylops* puparium. — Abbreviations: cph – cephalothorax of adult female *Stylops*, ep – empty puparium, p – intact puparium. Scale bar – 2 mm.

Declaration about Peer-Reviewed Publications

During final stages of thesis completion leading up to the thesis defence of August 30, 2019, two portions of Chapter 3 of this thesis had been submitted as manuscripts for peer-review publication. In both instances, these manuscripts have been accepted by the journal editors, shortly before submission in mid-September, 2019, of the revised thesis.

Specifically, a manuscript dealing with Sections 3.4.1 and 3.5.1 that examines the morphology of the adult male of *Stylops advarians*, was accepted on September 1, 2019 and currently is "in press" with *Zootaxa.* Copyright for this article has been transferred to the journal's publisher, Magnolia Press of Auckland, New Zealand.

Furthermore, Chapter 3 – Sections 3.4.4.1, 3.4.4.2, and parts of Section 3.5.4, which examine the morphology of the host-seeking first-instar larva of *Stylops advarians*, were compiled into a manuscript that was accepted on August 16, 2019 and subsequently slated to be published in the September, 2019 issue of volume 52 of *Arthropod Structure & Development*. It has been given this DOI designation: https://doi.org/10.1016/j.asd.2019.100881. Copyright for this article was transferred to the journal's publisher, Elsevier of Amsterdam, The Netherlands.

At time of submission of this revised thesis, both articles await their final page numbers.

All work reported in these two publications was done by me. The writing and editing was worked on by both my co-author (A.R. Davis) and me.

CHAPTER 3: STRUCTURE OF VARIOUS LIFE STAGES OF *STYLOPS ADVARIANS*

3.1 Abstract

Three stages of *Stylops advarians* were examined to understand the morphological and anatomical adaptations that enable them to be successful parasites. The adult male of *S. advarians* was described for the first time, highlighting its six-segmented antennae, elongated metathorax, hook-like aedeagus, large hind wings, and haltere-like fore wings. The adult female was neotenic and retained many larval characteristics, such as an unsclerotized abdomen. Females had two mandibles, a mouth opening, a brood opening, and a pair of spiracles on their cephalothorax. Female parasites were collected and sectioned still within the gaster of their *Andrena milwaukeensis* host. Those viviparous endoparasitic females collected in early May had many eggs packed together throughout their abdomens. Each female parasite collected later in the spring had many first-instar embryos at various developmental stages within its abdominal hemolymph. Fully developed first-instars were observed in the female's brood canal, as well as her cephalothorax, presumably travelling to her brood opening. Non-stylopized and stylopized *A. milwaukeensis* were sectioned to examine the impact that female parasites have on their host's anatomy. Stylopized female bees of *A. milwaukeensis* did not possess ovaries. There was little impact on the hindgut of stylopized hosts, but the crop was shifted away from the parasite. If two or three female parasites were present, the crop was restricted to a very small area, below the parasites, inhibiting its expansion. Female parasites were found to reside on one of their host's air sacs. If a third, central female was present, she was supported by the abdomens of the two parasitic females on either side of her. First-instar larvae were examined using scanning electron microscopy. Several sensory structures, such as olfactory pits and setae, were detected on the head. Coarse hairs called spinulae were located dorsally and ventrally on the thorax and

abdomen, presumably to help these larvae get onto an adult bee host. The pro- and mesothoracic tarsi were modified into flexible pads likely used for adhesion, whereas the metathoracic tarsi were stiff rod-like appendages likely best suited for movement. Abdominal segment 11 is split into two conical structures, each with one long caudal filament. The caudal filaments have a dilated tip at the end, which is likely used for adhesion. The nervous system of the first-instar is located in the thorax and is highly centralized. A digestive system is present in these young larvae, but likely not functional due to the junction of the midgut and hindgut being closed.

3.2 Introduction

Strepsiptera is an order of endoparasitic insects, best known for its unique life cycle, due to the extreme sexual dimorphism of the adult males and females. Adult males are free-living and have typical adult insect characteristics (Kathirithamby, 1989). Adult females, however, are neotenic, and are never free-living after their first-instar larval stage (except for those in the family Mengenillidae).

The sole job of male strepsipterans is to find a female with which to mate. Males have several adaptations that help them successfully find and copulate with an endoparasitic female. Males have large hind wings and haltere-like fore wings, the muscles of which are developed inside the puparium (Smith and Kathirithamby, 1984). The hind wings provide the males with powerful and versatile flight, whereas the halteres likely maintain balance (M. Hrabar, personal communication). Once the males emerge from their puparia, they are immediately ready to fly and search for a female (Kathirithamby, 2009). Males have well-developed antennae which are used for detecting the sex pheromone released by conspecific females (Tolasch et al., 2012; Cvačka et al., 2012; Lagoutte et al., 2013; Hrabar et al., 2015; Zhai et al., 2016). Once the

general location of a female is detected, the males use their large raspberry-shaped eyes to more accurately pinpoint the female, which resides inside her host. The eyes are unique among insects, having relatively fewer, but larger, lenses (Buschbeck et al., 1999). Buschbeck et al. (2003) suggests that when the males get close to a virgin female, the olfactory receptors may be too saturated by the pheromone, or the receptors react too slowly, and thus the eyes become extremely important for finding a female. Once a female is found, the male lands on her host, adhering using microtrichia on its tarsi (Pohl and Beutel, 2004), and begins the copulation sequence (Peinert et al., 2016). Male strepsipterans, like *Stylops*, with fast-flying hosts often have a hook-like aedeagus, which is useful for anchoring the male to the female, so contact is not lost during copulation (Peinert et al., 2016).

Most adult female strepsipterans are considered neotenic and continually reside inside their hosts, with only their cephalothorax visible externally. Females skip their pupal stage (Erezyilmaz et al., 2014; McMahon and Hayward, 2016), and metamorphose from the fourth-instar larva directly to the adult. The only visible change of this metamorphosis is the sclerotization of the cephalothorax, which is then pushed between two abdominal segments of the host (Kathirithamby, 2000). Female strepsipterans have no legs, eyes, antennae, external genitalia (except in the family Mengenillidae), or wings (Kinzelbach, 1971; Kathirithamby, 1989). Most of the abdomen of the female is unsclerotized and larva-like, and is protected within the host's body. Maeta et al. (2001) found that the females of *Pseudoxenos iwatai* were consistently found between the 3rd and 4th tergites of their host's abdomen, suggesting that occupying these locations gave the female the highest fitness.

The female's eggs are stored throughout her abdomen, and float inside the hemolymph (Pohl and Beutel, 2008; Peinert et al., 2016). Eggs hatch soon after being fertilized, and the

embryos develop into first-instar larvae inside the female (Fraulob et al., 2015). First-instar larvae use invaginations within the female's abdomen to access the brood canal. These invaginations of the adult female have been described as either an extra-genital duct system (Lange et al., 2013), or an apron (Kathirithamby, 2000). First-instar larvae travel through the brood canal and the female gives birth to the mobile first-instars through her brood opening (Kathirithamby, 2009).

The first-instar larva is the host-seeking stage and is arguably the most important stage of the strepsipteran life cycle. Few studies to date are devoted to an overall description of the morphology of the minute first-instar larvae of Strepsiptera, especially with respect to their adaptations for phoresy. The study by Pohl (2000) is by far the most comprehensive, as it examines 15 species of Strepsiptera throughout several families, including *Stylops melittae* Kirby collected from four *Andrena* species. Other studies focused on *Mengenilla chobauti* Hofeneder (Osswald et al., 2010) and *S. ovinae* (Rohnstein, 1953; Borchert, 1963; Knauthe et al., 2016; recorded as *S. muelleri* by Schneidereit, 1986). A recent review examines the effects of miniaturization on various adaptive features of these tiny strepsipteran first-instar larvae (Pohl and Beutel, 2019).

Several adaptations that these first-instar larvae use to find a host, which includes successful phoresis on an adult host bee in regard to *Stylops*, were examined. These adaptations include sensory structures, such as olfactory pits, stemmata, and several setae on the head (Knauthe et al., 2016), adhesive pro-and mesothoracic tarsi (Pohl, 2000; Beutel and Gorb, 2001; Pohl and Beutel, 2004), coarse hairs covering the thorax and abdomen dorsally and ventrally (Pohl, 2000), and potentially adhesive caudal filaments (Kirkpatrick, 1937; O'Conner, 1959; Borchert, 1963; Young, 1987).

The first-instar larvae of *Stylops* use a unique method of accessing a permanent host. Because *Stylops* infects an immature stage of *Andrena*, the first instars require an adult bee host to transport them to the bee's subterranean nest, where a permanent host can be found. The first-instar larvae of *Stylops* have been observed within the crops of non-stylopized andrenid bees (Linsley and MacSwain, 1957). Presumably, an andrenid will inadvertently ingest a first-instar, transport the larva to its nest, then deposit the parasite in a nest cell along with the regurgitated nectar and some pollen that the bee has also collected. The adult bee host soon lays an egg, which supplies the previously deposited larva with the permanent host that the young parasite needs to continue its life cycle (Linsley and MacSwain, 1957). The method of travelling within the adult bee host may be a relatively novel adaptation or a successful accident, as I hypothesize that the morphology of first-instar larvae of *Stylops* is better adapted for travelling on the surface of an adult host bee to the bee's nest. This hypothesis is supported by observations of Ulrich (1956), who found *Stylops* larvae on the outside of non-stylopized bees, as well as observations of *Stylops* first-instars in the pollen pellets of *Apis mellifera* (Bolchi Serini et al., 1996; D.J. Wiens and A.R. Davis, unpublished).

The anatomy of first-instar strepsipteran larvae is understudied, like the morphology. Rohnstein (1953) observed the tracheal system of the first-instar larvae of *S. ovinae*, but did not observe spiracles, so how oxygen passes into the tracheae is unknown. The nervous system of the first-instar larvae of *Stylops melittae* is highly concentrated to the thorax and abdomen (Pohl, 2000). The shift of the brain and other nervous system organs out of the head is also present in the basal strepsipteran genus, *Mengenilla* (Beutel et al., 2005), suggesting that this shift is a consequence of the minute size of the first-instar larvae (Pohl and Beutel, 2019). Knauthe et al. (2016) provide an extensive study on the anatomy of the head of *Stylops ovinae*, which includes

locations of the mandibles, muscles, and nerves. It was found that the setae and olfactory pits on the head were innervated, suggesting that these structures are important for responding to sensory stimuli (Knauthe et al., 2016).

A digestive system is present in the bodies of the first-instar larvae, even though they are not believed to feed (Cook, 2014). To gain nutrients while searching for a permanent host, these larvae likely use a yolk sac supplied by their mother (Giusti et al., 2007). The foregut begins at the ventral opening of the preoral cavity and leads to the midgut, which is located in the metathorax (Pohl, 2000). The midgut is closed to the rectum, and reaches to the sixth abdominal segment in the basal *Mengenilla chobauti*, and to the third abdominal segment in *Stylops melittae* (Pohl, 2000). The rectum is connected to the midgut, but is also closed off in the first-instar larvae (Pohl, 2000).

Using conventional microtechniques such as scanning-electron microscopy, and the sectioning and staining of fixed, intact or dissected tissues examined by light microscopy, the goals of this research chapter were to investigate the morphology and anatomy of three biologically significant life stages of *Stylops advarians* — the adult male, the adult female, and the host-seeking, first-instar larvae. Of particular interest were structural features of the parasite's life stages that reflected their adaptive abilities to associate with phoretic behaviour, leading to the parasitization of an immature stage of their host, a solitary bee species, *Andrena milwaukeensis*.

3.3 Methods

3.3.1 *Specimen Collection and Storage*

3.3.1.1 *Adult Males*

Adult males proved difficult to collect. From 2016-2018, I placed bees stylopized by an adult female, into cloth-mesh bags to attempt to lure males to her location via her presumed sex pheromone (Tolasch et al., 2012; Cvačka et al., 2012; Lagoutte et al., 2013; Hrabar et al., 2015; Zhai et al., 2016). No males were collected using this method. In 2018, I deployed malaise traps and nest emergence traps around the study site to attempt to collect males that were travelling towards virgin females. Malaise traps were placed near *Shepherdia*, the flowers of which *A. milwaukeensis* was observed foraging on in early May (see Section 2.3.1). I presumed the female parasites were within the early emerging, foraging bees, thus the males would fly towards stylopized bees when they were at the *Shepherdia* plants, similar to the observations of Linsley and MacSwain (1957). No males were collected within the malaise traps. Nest emergence traps were also placed over bee nests to attempt to acquire the stylopized bees before male *Stylops* emerged from their puparia. No *A. milwaukeensis* were collected using nest emergence traps. Finally, I used a sweep net to try and capture bees that still had intact puparia protruding from their abdomens. One such bee, with two puparia, was collected on May 14, 2018 (see Section 2.4.2).

The bee with two puparia protruding from its abdomen (see Fig. 3.1A) was initially kept in a small glass vial with a bright light shone on it to try and coax the males to emerge (James et al., 2016). The males remained within their puparia and once the bee died it was placed into a vial with 70% ethanol until dissection. An adult male (Fig. 3.1B) was removed from the puparium on the gaster's left side (Fig. 3.1A right) on October 23, 2018, using the method of

Kenner (2002). The other male specimen was removed on June 26, 2019 and used for molecular species identification (see Section 2.3.3).

3.3.1.2 *Adult Females*

Seventeen adult females were removed from their hosts for examination. Four of these females were destined for anatomical sectioning in resin, but were destroyed inadvertently during removal and were not examined further. One host bee had two females protruding from different tergites, and was dissected to determine if there were other females that did not protrude within (Linsley and MacSwain, 1957). Two females were dissected from their hosts and are destined for DNA sequencing, following defence of this thesis. One female was removed from its host and used to examine the first-instar larvae inside. This female was cut open and several living first-instar larvae emerged. The remaining eight females were used to examine the characteristics of the cephalothorax, such as the mandible tooth and mesothoracic band (see Section 3.3.2.1).

3.3.1.3 *First-Instar Larvae*

First-instar larvae of *S. advarians* were collected as they emerged from the brood opening of their mother's cephalothorax, beginning around May 24 each year. Many first-instar larvae were placed on aluminum stubs to be used for scanning electron microscopy (see Section 3.3.2.2). Other first-instar larvae were placed in 70% ethanol, and were sequenced molecularly to identify their species (see Section 2.3.3). Many emerged first-instar larvae remain on the abdomen of several stylopized bees. First-instar larvae were also collected by dissecting females and removing them from inside their abdomens.

3.3.2 *Specimen Preparation for Structural Investigation*

3.3.2.1 *Observations by Dissecting Microscopy*

Initial examination of the adult male was done using an Olympus SZ-ST dissecting microscope. Measurements of important features were taken using a Nikon Stereoscope SMZ-10, with a Dino-Eye USB digital colour camera attached, and DinoCapture 2.0 software.

Females of *S. advarians* were examined using a SZ-ST Olympus dissecting microscope. The patterns of colouration of the cephalothorax and the mandibles were examined to assist identification of the species (Pierce, 1918; Bohart, 1941).

Twelve non-stylopized *Andrena milwaukeensis,* collected after May 22, 2018 (the date of first appearance of emerged, first-instar larvae) were examined at 40X magnification for the presence of first-instar larvae on their bodies, including the pollen accumulated on their bodies, using an SZ-ST Olympus dissecting microscope. Twelve additional foraging bees were also dissected and examined to check for first-instar larvae within their crop (Linsley and MacSwain, 1957), using this same microscope.

3.3.2.2 *Scanning Electron Microscopy*

Adult males were not examined using scanning electron microscopy (SEM) as there were too few specimens to allow for such an examination.

Two adult females were used for SEM examination to try and identify species based on mandible morphology (Bohart, 1941). The females destined for SEM were first fixed with 2% glutaraldehyde in a 25mM sodium phosphate buffer. The females were post-fixed with 1% osmium tetroxide in the same buffer, for 1–2 hours. After rinsing with several changes of buffer and then distilled water, the samples in vials were gradually dehydrated in an ascending series of

acetone. Thereafter, the samples were placed in a Polaron E3000 Series II dryer and dried to the critical point. Once dried, the adult females were coated with gold using an Edwards S150B sputter coater. Finally, these females were imaged using a Phenom G2 Pure Scanning Electron Microscope.

Isolated, emerged first-instar larvae (n = 151) of *S. advarians* were adhered directly to aluminum SEM stubs using black sticky tabs (SPI Supplies, Toronto, Canada). Moreover, to allow examination of the body hairs of *A. milwaukeensis*, and any adhering pollen, the gasters of dead hosts (n = 5) each with a protruding cephalothorax of an adult female *S. advarians*, were mounted on SEM stubs also without any fixation, dehydration, or critical-point drying (Davis, 2009). These stubs were coated with gold using an Edwards S150B Gold Sputter Coater prior to examination using a Phenom G2Pure SEM or Hitachi SU8010 FE-SEM.

3.3.2.3 *Histological Techniques*

Four non-stylopized and 11 stylopized bees of *A. milwaukeensis* from early May to the third week of June (2016–2018) were chosen as representatives for anatomical investigation (Table 3.1), and were processed for resin sectioning. The gasters of these bees were removed and placed in 2% glutaraldehyde in 25mM sodium phosphate buffer. The glutaraldehyde was rinsed three times with the sodium phosphate buffer, then three times with distilled water before the dehydration process involving ethanol solutions replaced in an ascending order (10, 30, 50, 70, 85, 95, 100%), with each change lasting one hour. Each ethanol change was done once, except the introduction of 100% ethanol which was done three times, the last of which was left overnight. Specimens were then infiltrated gradually with a mixture of 100% ethanol and LR White resin (London Resin Company Limited, Basingstoke, England). Finally, I infiltrated the

specimens with fresh 100% LR White resin three times, with the final change occurring overnight. Each abdomen was transferred to a Beem capsule and polymerized in an incubator at 60 °C. Specimens were sectioned using a Reichert Ultracut microtome and glass knives. Sections were cut at 2 μm and stained using 1% toluidine blue 0 in a 1% sodium borate solution.

The abdomens of the four non-stylopized bees were sectioned to examine their anatomy (control) and compare it to the anatomy of stylopized bees. Two non-parasitized bees (specimens 1, 2) were sectioned in cross-section and two (specimens 3, 4) were sectioned longitudinally (Table 3.1). Of the 11 stylopized *A. milwaukeensis* bees, seven (specimens 5–8, 12, 13, 15) were sectioned in cross-section, three (specimens 10, 11, 14) were sectioned longitudinally, and one (specimen 9) was sectioned laterally (Table 3.1). Three of the 11 stylopized bees were infected with two female strepsipterans (specimens 12–14) and one of the 11 stylopized bees was infected with three female strepsipterans (specimen 15; Table 3.1). One stylopized bee (specimen 5) collected on May 3, 2017 was examined to observe the eggs within the female strepsipteran (Table 3.1).

3.3.3 *Imaging*

Sections were initially viewed and screened using an Olympus CH30 light microscope at 40–400X. Sections were imaged using a Zeiss AxioPlan microscope with an AxioCam105 mounted to the top. Images were captured with this camera, and the software ZEN 2 was used to adjust contrast and colours. Images were captured at 25–400X.

Table 3.1. Table showing how each host bee (n=15) of *Andrena milwaukeensis* was sectioned, the intensity of stylopization, and the date each bee was collected. Unless specified otherwise, each stylopized bee possesses a single adult female of *Stylops advarians*. Specimen numbers listed below correspond to those at the ends of captions for Figs. 3.13–3.18, and 3.23.

Specimen Number	Date Collected	Specimen and Section Type
1	May 31, 2016	Non-stylopized bee in cross section
2	June 19, 2018	Non-stylopized bee in cross section
3	June 18, 2018	Non-stylopized bee in dorsal-ventral section
4	June 18, 2018	Non-stylopized bee in dorsal-ventral section
5	May 3, 2017	Stylopized bee in cross section (eggs present)
6	June 5, 2017	Stylopized bee in cross section
7	June 7, 2017	Stylopized bee in cross section
8	June 13, 2017	Stylopized bee in cross section
9	June 14, 2018	Stylopized bee in lateral section
10	June 3, 2016	Stylopized bee in dorsal-ventral section
11	June 8, 2016	Stylopized bee in dorsal-ventral section
12	May 4, 2018	Stylopized bee (2 females) in cross section
13	June 18, 2018	Stylopized bee (2 females) in cross section
14	June 12, 2017	Stylopized bee (2 females) in dorsal-ventral section
15	June 13, 2018	Stylopized bee (3 females) in cross section

The first-instar larvae were initially examined using a Phenom G2Pure SEM, before using a Hitachi SU8010 field emission scanning electron microscope (FE-SEM) to capture the more minute structures of the first-instars.

3.4 Results

3.4.1 *Morphology of Adult Male*

After dissection liberated a single dead adult male from its puparium (Fig. 3.1A right), this specimen (Fig. 3.1B) was observed, described, and measured as follows, based on the features included in the study of a male strepsipteran by Roy and Hazra (2016).

Total length 2.69 mm; length of the metathorax 1.79 mm; width of the metathorax 0.64 mm; length of the antenna 0.69 mm.

Colour. Head deep brown, thorax deep brown, abdomen light brown, and hind wings clear.

Head elongated laterally; length 0.28 mm, width 0.61 mm. Eyes prominent, 49 ommatidia per compound eye when viewed ventrally. Antenna six segmented (Fig. 3.22), antennomere I 0.13 mm long, antennomere II 0.05 mm long, antennomere III enlarged as a flabellum 0.52 mm long, antennomere IV 0.26 mm long, antennomere V 0.14 mm long, antennomere VI 0.11 mm long. Maxillary palp (Fig. 3.3) curved anteriorly and over twice as long as its base. Maxillary base 0.15 mm long; palp 0.37 mm long. Mandible (Fig. 3.4) 0.17 mm long, beveled at tip, less than half the length of the maxillary palp.

Metathorax (Fig. 3.5). Metaprescutum slightly rounded anteriorly, separated from the prescutum by a depressed scutal area; prescutum with three scutae rounded anteriorly, protruding from the scutum, prescutum 0.42 mm in length and 0.64 mm in width; scutellum long and curved anteriorly, 0.27 mm in length; postlumbium almost as long as wide, slightly constricted near anterior end, 0.40 mm in length

and 0.36 mm in width; postnotum constricts at the postlumbium, but widens posteriorly, and is rounded at the posterior end, postnotum 0.70 mm long and 0.52 mm wide.

Fore Wing (Fig. 3.6). The fore wing 0.65 mm long, continually broadening at apical end.

Hind Wing (Fig. 3.7). Hind wing with two detached veins – R_2 and R_3, R_2 slightly shorter than R_3, R_3 almost touches the wing margin; R_3 located above the apex of R_4. MA is slightly shorter than CuA_1; CuA_1 nearly touches the wing margin. CuP is just over two-thirds the length of CuA_1.

Legs (Figs. 3.8–3.10). Fore coxa 0.33 mm long, fore femur 0.37 mm long, fore tibia spatulate and 0.30 mm long, fore tarsomere I slightly curved and 0.08 mm long, tarsomere II 0.13 mm long, tarsomeres III and IV 0.14 mm long. Mid coxa 0.46 mm long, mid femur 0.33 mm long, mid tibia 0.35 mm long, mid tarsomere I 0.08 mm, tarsomeres II, III, and IV are equal in length at 0.12 mm. Hind coxa 0.31 mm long, hind femur 0.40 mm long, hind tibia 0.43 mm long, hind tarsomere I 0.12 mm, II 0.13 mm, III 0.12 mm, and IV 0.11 mm long. Ventral surface of last tarsomeres IV of each leg hairy without claws.

Abdomen. Abdomen sclerotized, 10-segmented.

Aedeagus (Fig. 3.11). Aedeagus 0.35 mm long with terminal hook 0.12 mm long, at ca. 67° angle to shaft; shaft almost two times longer than the terminal hook, shaft thin, 0.23 mm long, terminal hook beveled.

3.4.2 *Structure of Adult Female*

3.4.2.1 *Morphology*

Adult female *Stylops advarians* have a similar body plan to females of other *Stylops* species, as well as other strepsipteran genera and families. The female is often characterized as neotenic, meaning she retains her larval features as an adult. No eyes, legs, wings, antennae, or

external genitalia were found. Her body is 4.5 mm long (Fig. 3.12A). The only parts of the body that are sclerotized are the cephalothorax and the first abdominal segment, which are also the only parts of the female's body visible externally while she is inside her host. The rest of the female's abdomen is soft and unsclerotized. Segments II–VI on the dorsal side of the abdomen are discoloured, and show the location of the brood canal region (Kathirithamby et al., 2015) (Fig. 3.12A).

The cephalothorax is the only part of the female used to identify species when no male is present, as the abdomen does not have any identifiable features (Pierce, 1918; Bohart, 1941). The morphology of the mandibles (Fig. 3.12B, black arrow, E), including tooth size and position (Fig 3.12D,E), and the colouration pattern of abdominal segment I (Fig. 3.12A,B, white arrow) were often used to describe species of *Stylops* (Pierce, 1918; Bohart, 1941). Using the identification guide by Bohart (1941), I thought that my species could be one of four possibilities based on my interpretation of the colouration pattern and mandible morphology: *Stylops duboisi*, *S. erigeniae*, *S. grandior*, or *S. polemonii*. Molecular analysis of our species revealed that our specimens (first-instar larvae; adult male) were actually *Stylops advarians*.

The female has two spiracles, which were also used for identification (Pierce, 1909), posterolaterally on the cephalothorax (Fig. 3.12D,F). There are no abdominal spiracles. On the ventral side of the female's cephalothorax there is a line showing where the head and thorax are fused (Fig. 3.12C). No other features were observed on the ventral surface of the cephalothorax of *S. advarians* (Fig. 3.12C), unlike females of *Xenos peckii,* which have vertical fold lines (Hrabar et al., 2014).

Only the cephalothorax of the adult female *Stylops* can be seen protruding from the host. Anteriorly the cephalothorax has mandibles, a mouth opening, and a brood opening (Fig. 3.12B),

but the rest of her body is hidden beneath the host's tergites and within her host's abdomen (Fig 2.6A–C).

3.4.2.2 *Histology*

The adult female strepsipteran is surrounded by two previous larval exuvia (Löwe et al., 2016; Richter et al., 2017) (Fig. 3.13A), because the larval instars do not shed their exuvia after their first moult (Kathirithamby et al., 1984). The adult female is considered a 'bag of eggs' (Kathirithamby et al., 1990b; Kathirithamby, 2018). Upon creating an incision in several female abdomens, many eggs proceeded to spill out of the opening. Sections show these eggs tightly packed together in the abdomen (Fig. 3.13B,C), residing in the hemolymph (Peinert et al., 2016).

Once the eggs are fertilized, they hatch a short time later and the embryos develop into first-instar larvae, still within the female's hemolymph. First-instar larvae of different developmental stages can be seen within the female's abdomen (Fig. 3.13D), due to asynchronous development (Kathirithamby, 2009). Once the larvae are fully developed, they begin travelling to the brood canal, which is entered from the hemolymph through invaginations (Kathirithamby, 2000; Lange et al., 2013). Fig. 3.13E shows these first-instars within the brood canal of their mother. The brood canal leads to the cephalothorax, and first-instars can often be seen within their mother's cephalothorax as they travel within the brood canal (Fig. 3.13F) towards her brood opening (Fig. 3.12B,D).

3.4.3 *Impact of Parasitism on Host Bee Abdomen*

Examination of non-stylopized *Andrena milwaukeensis* showed that the bee's abdominal organs occupy the entire abdomen. Parts of the digestive tract were found to be centralized

within the abdomen (*i.e.* not shifted to the left or right side). The oesophagus is present anteriorly (Fig. 3.14A) and leads to the crop (Fig. 3.14A). Organs of the digestive system, such as the crop (honey sac), midgut, and rectum, are present anteriorly to posteriorly in the abdomen (Fig. 3.14B). The crop is often observed full of pollen (Fig. 3.14C,E) and when full, takes up a large portion of the abdomen. The crop leads to the midgut via the proventriculus (Fig. 3.14C,E), which is found posterodorsally (Fig. 3.14C,E). The midgut is connected to an often exine-filled rectum, via the ileum (Fig. 3.14F). Eggs were present upon dissection of several non-stylopized bees (Fig. 3.14D,E). Two large air sacs are present anteriorly and laterally within the abdomen, and flank the components of the digestive tract, such as the crop and the midgut (Fig. 3.14G).

When one adult female of *Stylops advarians* is present in the abdomen of *A. milwaukeensis*, the host's internal organs were not centralized, and instead were found shifted to the side opposite of where the female parasite resides (Fig. 3.15A,E), as well as below the parasite (Fig. 3.15A,F). Fig. 3.15B shows the area that the female parasite occupies within her host's abdomen, when all of the host's organs are removed. The posterior end of the host bee's abdomen is less affected than the rest of its abdomen, as the female parasite's body is not within the host's until after the host's fourth segment (Fig. 3.15F). The host's midgut and rectum are more tightly squeezed into the posterior end, but they are not shifted laterally (3.15A,F). Near the posterior end of the bee, the female's cephalothorax resides on the tergite of her host (Fig. 3.15C,F). When the female parasite's body is inside her host, it is supported by one of her host's air sacs (Fig. 3.15D,G). No reproductive structures or eggs belonging to the host bee were observed.

When two female parasites are present in the abdomen of *A. milwaukeensis*, the fore- and midgut cannot be shifted away from one female due to the presence of the second. Instead, these

organs are squeezed below the two parasites (Fig. 3.16A) and towards the posterior end of the bee's abdomen (Fig. 3.16B). The crop is restricted by the presence of the two parasites, and is unable to expand. The expansion of the crop may also be restricted by air sacs on either side of it (Fig. 3.16B,D). The posterior end of the bee remains relatively unaffected as the cephalothoraces (Fig.3.16E), plus the first abdominal segments of the two females, are not within the host at its posterior end (Fig. 3.16C). Air sacs look to be present on either side of the midgut (Fig. 3.16C). The abdomens of the female parasites are likely supported by the air sacs of their host (Fig. 3.16D). No reproductive structures or eggs were observed inside the host bee's abdomen.

Bees of *A. milwaukeensis* infected by three females show similar results to those infected by two. The third female occupies the centre of the host's abdomen, with the other two females on either side of it (Fig. 3.17A). Three female parasites take up more space inside the host bee's abdomen than when two females are present, but the organs are similarly shifted to underneath the abdomens of the three parasites (Fig. 3.17B,C). The females that occupy the left and right side of the host's abdomen are supported by the host's air sacs (Fig. 3.17B,C). The central female seems to be supported by the females on either side of her (Fig. 3.17B,C). The digestive system is reduced (Fig. 3.17C), similar to bees stylopized by one or two female parasites. No reproductive structures or eggs were observed inside the host's abdomen.

3.4.4 *Structure of First-Instar Larvae*

3.4.4.1 *Morphology*

Hundreds of first-instar larvae of *Stylops advarians* were found to emerge from the brood opening of the cephalothoraces of endoparasitic adult females, and were highly mobile.

Each first-instar larva was prognathous, light brown and sclerotized over the entire body (Fig. 3.18A,B,D). Maximum body length and width were 159 µm and 67 µm, respectively. Two caudal filaments at the abdomen tip measured to around two-thirds of the body length (Fig. 3.18A). Maximum caudal filament length was 106 µm.

The dorsal region of the forward-projecting head of the first-instar larva of *S. advarians* had a slight curvature toward the larva's ventral side and possessed several sensory structures. Two prominent eye spots occurred on the head capsule posterolaterally (Fig. 3.18B). A region of stemmata was visible dorsolaterally, but the prothoracic segment covered all but the first (anterior) one (not shown).

The head capsule had five pairs of setae, of which three pairs inserted dorsally (Fig. 3.19A,B). Each external eye seta inserted near the prothoracic segment, but medially to the region of stemmata comprising each eye spot (Fig. 3.19A). Each posterior marginal seta, although near an external eye seta, occurred closer to the ventral side (Fig. 3.19B) since margins of the head capsule curve down. Each anterior marginal seta occupied the midline region between the head's dorsal and ventral integument, and was in line with each maxillary palp (Fig. 3.19B). On the other hand, each frontal seta was found just medial to each olfactory pit (Fig. 3.19A). Ventrally, each maxillary seta was slightly posterolateral to each maxillary palp (Fig. 3.19B).

An olfactory pit occupied the dorsal surface posterolaterally to each frontal seta (Fig. 3.19A). There were two distinct depressions evident per pit on each side of the head, with 10–14 sensilla in each depression (inset of Fig. 3.19A).

The lip-like structure evident at the anterior portion of the head (Fig. 3.19A) could not be seen from the ventral side (Fig. 3.19B). An opening between the lip-like structure and the

medially fused maxillae ventrally that would allow mandibles to extrude, was not apparent (Fig. 3.19B), nor were mandibles detected by SEM, contrary to first-instar larvae of *S. ovinae* (Knauthe et al., 2016). However, anatomical sections revealed that a pair of mandibles exist (inset of Fig. 3.18D).

Vestigial mouthparts were present on the head capsule's ventral surface. A pair of maxillary palps were found anterolaterally to the maxillary setae and inserted directly posterior to the maxillae (Fig. 3.19B). Moreover, a labium was present and covered the ventral opening of the preoral cavity (Fig. 3.19B). We did not observe any instance of the anterior portion of the labium being open, as observed by Knauthe et al. (2016), but a fissure was noticeable in this region.

Ventrally, the head capsule elongated posteriorly (Figs. 3.18C right; 3.19B) as large lobes (labelled as postgenal bridges by Pohl (2000) and Knauthe et al. (2016); however, the use of this particular label is uncertain based on location of these bridges in other insect groups) to partially cover the prothorax (Fig. 3.19B). A gap formed at the midline before the lobes connected at the preoral cavity, below the labium, thereby revealing the cervix (Fig. 3.19B).

The prothorax was 1.5 times longer than the mesothorax, and the latter about twice as long as the metathorax (Figs. 3.18A,B,D; 3.20A). Dorsally, the prothorax covered a portion of the head capsule (Fig. 3.19A), and the lateral margins extended downward (Fig. 3.20B), lending a concave nature to the body ventrally. This ventral concavity, however, is likely a drying artifact, since the larva's body is more cylindrical in nature (Knauthe et al., 2016; Pohl and Beutel, 2019). Also dorsally, two pairs of setae occurred per thoracic segment (Fig. 3A,B); one pair was located on either side of the body's midline (Fig. 3.20A), whereas the other pair was situated laterally (Fig. 3.20A,B arrows).

The three-segmented thorax also featured many coarse hairs (spinulae) on the posterior margins of both the body's dorsal (Fig. 3.20A) and ventral (Fig. 3.20B) surfaces. Spinulae were directed to the posterior end of the larva and laid across the next body segment (Figs. 3.18A,D; 3.20A,B). On the prothorax dorsally, spacing of the spinulae did not exhibit a clear pattern; however, in contrast to the other thoracic segments, spinula length was relatively short (Fig. 3.18A), especially near the body's midline (Fig. 3.20A). Compared to the prothorax, spinulae on the mesothorax were more numerous and longer (Fig. 3.18A), with the relatively longer spinulae not evenly spaced, but often arising as couplets (Fig. 3.20A). Spinulae were longest, however, on the metathorax (Fig. 3.18A), where they typically displayed a pattern of one long, followed by two short, spinulae (Fig. 3.20A). Occasionally, one or three short spinulae were found between two long ones. Often, the long and short spinulae that alternated per segment dorsally were not spaced equidistantly nor in precise alignment with those of the adjacent segment that spinulae overlapped (Fig. 3.20A).

In contrast to the dorsum, spinulae on the ventral surface of the three thoracic segments were fewer, shorter, and more evenly spaced per segment where they occurred on the posterior edges of elongated sternal plates (Fig. 3.20B). Variable among individuals, each plate possessed 5-7 spinulae. The trend for increased spinula length from pro- to metathorax was also evident, ventrally (Fig. 3.20B).

Especially owing to attachment of the legs, overall the thorax was more complex ventrally, than dorsally. The three sternal plates extending posteriorly were sclerotized, rigid and straight (Fig. 3.20B) and not only separated the two coxae per thoracic segment, but by overlap, also intervened anterior regions of the coxae of the segment posterior (Figs. 3.18C right; 3.20B).

A pair of setae occupied each sternal plate, one seta to each side of the body's midline (Fig. 3.20B).

Each leg was a highly specialized appendage, being comprised of a coxa, trochanterofemur, tibia, and a clawless, single-jointed tarsus (Pohl, 2000; Pohl and Beutel, 2004). The number of spinulae per leg segment varied between individuals, but the number of setae remained consistent, with four on each of the coxae and one on each of the trochanterofemora (Fig. 3.20C,D). The coxae had straight setae (Fig. 3.20C), whereas the setae of the trochanterofemora were curved (Fig. 3.20D). The pro- and mesothoracic tarsi have a sclerotized dorsal plate, and a prominent spur on the metathoracic tarsus (Fig. .203E-G). This plate was longest, and occasionally forked, on the mesothoracic tarsus (Fig. 3.20F), but shorter and simple on the pro- and metathoracic tarsus (Fig. 3.20E,G). The specialized pro- and mesothoracic tarsi were flattened and widened to a paddle-like shape, with flexibility to adhere to a substrate (Fig. 3.20E,F), including the tergite of an adult bee (Fig. 3.22A top right) and the surface of a pollen grain (Fig. 3.22B). On the other hand, the metathoracic tarsus was elongate and cylindrical, but abruptly dilated terminally (Fig. 3.20G). Contrary to the saccate nature of the tarsi on the pro- and mesothoracic legs, the cylindrical metathoracic tarsi with their short flattened distal regions appeared rigid and able to push, and thereby support, the larva (Fig. 3.22B right).

Similar to the thorax, at its lateral regions the dorsal side of the abdomen curved around to connect with the ventral side, giving the larva an overall concave appearance (Figs. 3.18A; 3.21A,B). Manifestation of this body curvature was evident by the formation of an air bubble trapped ventrally, near the thoracic-abdominal interface, in specimens mounted for photography (Fig. 3.18B). Again, this concavity may represent a drying artifact. However, the clear dorso-

ventral compression of the body (Fig. 3.18A,D) allowed multiple liberated larvae to partially occupy inter-tergite regions of their mother's host bee, for example (Fig. 3.22A).

Both dorsally and ventrally, each of the nine apparent abdominal segments overlapped with the segment posterior to it. These segments were distinguishable from those of the thorax by their absence of setae (Fig. 3.21A,B). Generally, the dorsal abdominal segments 1–7 were shorter in length than the metathorax. Segments 1–3 had similar width and length (Fig. 3.21A). Segments 5–7 had lengths comparable to segments 1-3, but were of shorter widths as the abdomen began to taper posteriorly at segment 4 (Fig. 3.21A). That segment was narrower (about 0.8 the length) than segments 3 and 5 attached to it (Fig. 3.21A).

Ventrally, segment 1 was narrower than all other abdominal segments, despite being partially covered by the metasternal plate (Figs. 3.18C right; 3.21B). This connection between the thorax and abdomen (where the air bubble resides in Fig. 3.18B), as well as the junction of segments 5 and 6, appeared in various larvae as points of abdominal articulation (Fig. 3.18A). This assertion of a common point of inflection is supported also by the difference in the plane of sectioning between adjacent segments 5 and 6, evident in the tangential longitudinal section of a developed larva still within its mother (Fig. 3.18C right). However, in another larva, articulation is evident between segments 4 and 5, instead (Fig. 3.21B).

Anatomical sectioning revealed that, internally, the body of first-instar larvae contained an elongated, saccate structure (Fig. 3.18C) that was fairly central (Fig. 3.18C left,D) and began to enlarge within the mesothorax and continued at least to abdominal segment 3 (Fig. 3.18D). This structure is interpreted as a portion of the digestive tract's midgut.

Similar to the thorax, both the dorsal and ventral surfaces of the abdomen were covered in numerous spinulae which pointed towards the larva's posterior end and overlaid the next

abdominal segment (Figs. 3.18A,D; 3.21A,B). A pattern of each long spinula separated by two short spinulae was common on each of segments 1-8 (Fig. 3.21A,B). All spinulae on the dorsal surface are countersunk, whereas only the short spinulae are countersunk on the ventral surface. However, on segment 9, there was an alternation of long and short spinulae (Fig. 3.21A,B). A suture line indicated that segments 9 and 10 were fused (Fig. 3.21A), with the anus located posteriorly on segment 10 (Fig. 3.21A,B). A pair of setae are also present laterally on segment 9 (Fig. 4A,B). The terminal segment 11 was separated into two conical structures, each surrounding a long caudal filament (Fig. 3.21A,B) that gradually tapered to 0.25 μm nearing its tip (Fig. 3.21A-C). However, the tip of each caudal filament abruptly widened to a pad of about 2 x 1.25 μm (Fig. 3.21C,D) that evidently could adhere to substrates (Fig. 3.21D). A short seta is present along each caudal filament (Fig. 3.21A,B). Caudal filaments were commonly held straight (Fig. 3.22B) but were flexible (Fig. 3.22A top left) and evidently delicate; their absence (Fig. 3.22A left) is interpreted as having broken off, possibly as an artefact during specimen handling.

3.4.4.2 *Examination of Adult Bees of A. milwaukeensis for First-Instar Larvae of S. advarians.*

One first-instar larva of *S. advarians* was found within the crop contents (Fig. 3.22D,E) of the twelve non-stylopized bees investigated.

Of the twelve pollen-laden, non-stylopized bees of *A. milwaukeensis* examined, also a single larva of *S. advarians* was encountered on a bee surface. Specifically, it occurred within an aggregation of pollen on the hind leg (Fig. 3.22C) and was confirmed in isolation, by light microscopy (Fig. 3.18B).

Although only one first-instar larva was detected externally on non-stylopized adult bees taken in the field, SEM examination of several *S. advarians* larvae residing on the abdomens of stylopized bees after issuing from their protruding mothers, gave insights into how larvae may travel externally on an adult bee. Some larvae were partially cryptic after creeping head-first between the host's overlapping tergites (Fig. 3.22A left) in a manner unopposed to the natural orientation of the larval body's spinulae. Additionally, spinulae of the larval abdomen (Fig. 3.22A left) or prothorax (Fig. 3.22A right,B) engaged the host's plumose body hairs in a manner that evidently enhanced lodging on the host. Finally, adherence of the flexible pro- and mesothoracic tarsi apparently allow larvae to cling directly to the host's tergites (Fig. 3.22A top right) or to pollen sticking onto the host's body (Fig. 3.22B).

3.4.4.3 *Anatomy*

Anatomical sectioning revealed that, internally, the body of first-instar larvae contained an elongated, saccate structure (Fig. 3.18C) that was fairly central (Fig. 3.18C left,D) and began to enlarge within the mesothorax and continued at least to abdominal segment 3 (Fig. 3.18D). This structure is interpreted as a portion of the midgut.

The anatomy of first-instar larvae of *Stylops advarians* is very similar to that of the first-instars of *S. melittae* (Pohl, 2000). *Stylops advarians* first-instars have a digestive system, made up of an oesophagus, midgut, and hindgut. The hindgut is connected to the midgut, but this connection is closed off (Fig. 3.23A), an observation also seen in *S. melittae* (Pohl, 2000).

The nervous system is likely shifted from the head to the thorax of the first-instars of *Stylops advarians*, as seen in the basal *Mengenilla* and in *Xenos vesparum* (Beutel et al., 2005). Structures similar to the ganglia observed by Pohl (2000) were found dorsally and ventrally in

the mesothorax of *S. advarians* (Fig. 3.23B). These ganglia were found around the anterior end of the midgut (Fig. 3.23B).

3.5 Discussion

3.5.1 *Adult Male*

This male is included in the genus *Stylops* of the family Stylopidae, based on the six-segmented antennae with an enlarged antennomere III, a large postlumbium, a hook-like aedeagus, and a scutellum that is at least as long as the prescutum (Bohart 1936; Bohart 1941; Kinzelbach 1978). These puparia were found in an *Andrena* bee, the recognized host of *Stylops* (Kathirithamby 1989), further supporting our classification. Our male specimen resembles *Stylops leechi* Bohart (1941) as the metathoracic scutellum reaches close to the prescutum, and the postlumbium is longer than it is wide, but differs because the fourth antennal segment is not more than twice as long as the fifth. Our specimen does not closely resemble *S. shannoni* Pierce or *S. childreni* Gray, the other known Canadian *Stylops* species for which adult males have been described (Pierce 1918; Griffith 1832). The differences between *S. advarians* and the males of those two species include the lengths of the antennomeres, and the morphology of the metathorax. In *S. advarians*, the length of antennomere IV is twice as long as antennomere VI, whereas in *S. shannoni* antennomere VI is slightly shorter than IV. In *S. advarians*, antennomere V is slightly longer than antennomere VI, whereas in *S. childreni* antennomere IV is longer than V. The scutellum of *S. childreni* is circular, whereas the scutellum of *S. advarians* is elongated and rounded anteriorly.

Using molecular analysis, we confirmed that this male specimen matches material identified as *Stylops advarians* (Balzer et al., unpublished), the same species ascribed by molecular methods to the first-instar larvae collected independently and subsequently described

(Balzer and Davis, 2019) at this same field site. Due to the extreme sexual dimorphism of the adults, and the cryptic nature of Strepsiptera (Kathirithamby *et al*. 2015), it is useful to use molecular tools to accurately identify strepsipteran species, and to match adult males with adult females and with the liberated, host-seeking first-instar larvae of the same species.The paratype male is stored in 70% ethanol and will be sent to the Royal Saskatchewan Museum (Catalogue No. RSKM_ENT_E-216645) in Regina, Saskatchewan.

3.5.2 *Adult Female*

The accuracy of identifying Strepsiptera morphologically to species has come under scrutiny following the study of Straka et al. (2015), which found that many *Stylops* species were misidentified. Due to the cryptic nature of the neotenic females and free-living males (Hayward et al., 2011; Kathirithamby et al., 2015), it is extremely difficult to use morphology alone to accurately identify a female strepsipteran to species. Molecular identification is likely the only reliable method for species identification of Strepsiptera.

The ventral side of the cephalothorax of female *Stylops advarians* did not show any fold lines, like those of *X. peckii* (Hrabar et al., 2014). The lack of these fold lines suggests that, unlike *Xenos*, the females of *Stylops* do not inflate their cephalothoraces to help disperse their sex pheromone. This conclusion corroborates the observations of Peinert et al. (2016), who saw no inflation or movement when observing pheromone-producing virgin females of *S. ovinae*.

Adult females of *S. advarians* were consistently found between the fourth and fifth tergites of their host, *Andrena milwaukeensis*. This position likely gives female parasites the most room to grow and develop, thus allowing more larvae to be produced within their abdomens (Maeta et al., 2001). The lateral positioning of the females may also be advantageous,

as the host's air sacs are likely used to support the body of a female *Stylops*. The few male puparia that were collected were also detected between the fourth and fifth tergites of the host bee (with the exception of a 2019 specimen; see Fig. 2.6E and Section 2.4.3), unlike another stylopid species, *Pseudoxenos iwatai* (Maeta et al., 2001), which has puparia protruding in the tergites above where females were typically found.

My findings on the anatomy of the adult female are similar to previous studies. The bodies of adult female *Stylops advarians* are surrounded by two previous larval exuvia, due to 'apolysis without ecdysis' (Kathirithamby et al., 1984). The second-larval instar exuvium remains the outermost exuvium in the adult stage, as these females do not form a puparium using their retained exuvia, as seen in males (Erezyilmaz et al., 2014; McMahon and Hayward, 2016). *Stylops advarians* females are full of eggs. Each female of other families of Strepsiptera produce eggs ranging from 1,000 (Miyamoto and Kifune, 1984, as cited by Cook, 2014) to 750,000 (O'Conner, 1959), leading to many larvae being produced. The number of eggs that *Stylops* females produce is currently unknown. This large production of offspring is critical to successfully having an offspring that finds a host, as the chances of one larva finding a host are evidently extremely small. Having more offspring increases the chances that at least one larva will successfully find a host and continue its life cycle.

The larvae within the adult female develop asynchronously (Kathirithamby, 2009), perhaps to take advantage of the prolonged foraging period of their host, *A. milwaukeensis*. If all larvae emerged at the same time, they would be deposited on very few flowers, and could overwhelm the adult bee host or permanent host, which would lead to the death of the strepsipteran larvae. When larvae emerge over an extended period of time, they will be deposited

on numerous flowers in several areas (see Section 2.4.2), thus increasing the chances that at least one of the female's offspring will travel with an adult bee host.

I was not able to find what has been described as the extra-genital duct system (Lange et al., 2013), or the apron (Kathirithamby, 2000), in my sections. However, I was able to find first-instar larvae within the brood canal of their mother. This location of the larvae suggests that these first-instars have a method for travelling from their mother's hemolymph, where they develop, to the brood canal. It is likely that *S. advarians* first-instar larvae use invaginations to get into the brood canal, and I may have missed them in my sections. The first-instars are thought to move through the brood canal to the cephalothorax (Kathirithamby et al., 2015), where they then emerge from the brood opening to be deposited onto a flower, and await an adult bee host with which to travel.

3.5.3 *Impact of Parasitism on Host Bee*

The most significant impact of *S. advarians* females on *A. milwaukeensis* is the loss of the host's reproductive organs. Strepsiptera have long been known to inhibit the development of the reproductive organs of their host (Pérez, 1886; Smith and Hamm, 1914; Kathirithamby, 2009; Cappa et al., 2014), and I found the same results in stylopized *A. milwaukeensis*. Non-stylopized bees had distinct eggs present, whereas stylopized bees had no sign of any reproductive organs, except the ovipositor, which now only functions as a sting.

Since only female bees were examined anatomically, the impact of *S. advarians* on male *A. milwaukeensis* reproduction is unknown. Smith and Hamm (1914) write that there was little impact on the testes of stylopized male bees of *A. nigroaenea*. Presumably, the testes require less nutrients than the larger ovaries, and thus they are not affected by the presence of a *Stylops*. A

similar result was found in stylopized male paper wasps (*Polistes dominula*), which were unaffected by their strepsipteran parasite (Cappa et al., 2014). Stylopized male wasps were still observed to maintain their sexual behaviour, and their reproductive organs were not inhibited by the parasite (Cappa et al., 2014). Further studies could be done to determine whether the number of *Stylops* within a male bee affects the development of the testes and sperm production.

The presence of one or more female *Stylops* likely impacted the amount of pollen and nectar stylopized bees could store in their crops. When one female is present per host, the crop apparently still has room to expand upwards, as well as laterally. When two or three female parasites are present, the capacity of the crop's expansion is heavily restricted; thus, food intake and storage is limited. The midgut and rectum of stylopized bees did not seem to be greatly affected. These digestive organs may be squeezed further into the posterior end of the bee, but because the female parasite's body is not inside her host's in this area, these organs are likely still able to expand to their normal size and complete their normal functions. Additionally, the loss of the stylopized female bee's reproductive organs may provide additional space for the mid- and hindgut to expand within the bee's gaster.

3.5.4 *First-Instar Larva*

The head of the first-instar larva of *Stylops advarians* had several sensory structures on both the dorsal and ventral side. The setae and olfactory pits were hypothesized to be sensory by Schneidereit (1986), which was confirmed by the presence of nerves connecting them to the larva's brain (Knauthe et al., 2016). The olfactory pits are likely chemosensory structures. We found that each of the two olfactory pits per larval head comprised two adjacent depressions in *S. advarians*, like in *S. ovinae* (Knauthe et al., 2016). However, each depression per olfactory pit in

S. advarians contained 10–14 sensilla, unlike just three in *S. ovinae* (Knauthe et al., 2016). The setae are likely mechanosensory. We did not find external antennae on *S. advarians*, and Knauthe et al. (2016) suggests that there are no internal remnants either. *Stylops* have three stemmata per eye spot (Pohl, 2000), but we never observed more than one per side of the head owing to suspected blockage of additional stemmata by the overlapping prothorax. Using light microscopy, the locations of the stemmata were observed as dark pigmented regions on either side of the head (Buschbeck, 2005).

Mandibles were not observed with SEM images of the first-instar larva. However, we did observe mandibles in sections of the first-instars. Mandibles are likely used to enter their permanent host's body, which for *Stylops* may be the egg (Linsley and MacSwain, 1957; Knauthe et al., 2016). Linsley and MacSwain (1957) observed the first-instar larva of *Stylops pacifica* entering the egg of *Panurginus melanocephalus.* The egg infecting hypothesis is supported by the method of how *Andrena* larvae receive their food. Solitary bees, like *A. milwaukeensis*, are mass provisioners, and will only lay an egg in a nest cell once there is a sufficient amount of food for the larva to feed on and then pupate. Since first-instar larvae travel with the nectar and pollen, they may access the nest cell before a host is deposited. As a means to increase their survival, *Stylops* first-instars may have adapted to burrow into the host egg, rather than wait for the egg to hatch into a larva. Knauthe et al. (2016) also hypothesize that first-instar *Stylops* larvae enter the host egg based on the structure of the ventral plate and ventral head opening. These structures allow attachment to a host's smooth surface using suction, thereby stabilizing the larva's head and allowing its mandibles to cut an opening into the host egg's chorion (Knauthe et al., 2016).

Though mouthparts are present, there is no evidence to suggest that they are used by the first-instar larvae to feed while they search for a host. The mouthparts on the head, such as the labium, maxillary palps, and maxillae, are vestigial. The mandibles have dentition (Knauthe et al., 2016), but are likely only used for cutting the host's cuticle or chorion before burrowing inside. A digestive system is present, leading from the preoral cavity on the ventral side of the head to the anus, but is likely not yet functional. At this first-instar stage, the midgut and rectum do not seem to connect to each other; instead, the junction of these parts of the digestive tract is closed (Pohl, 2000; Z. Balzer, unpublished). The first-instars of *Stylops* likely use yolk material present in their bodies, similar to *Xenos vesparum* (Giusti et al., 2007).

The spinulae and setae of the thorax may be used to help a first-instar larva get picked up by their phoretic host, stay on the phoretic host, or cling onto the pollen the phoretic host has collected. The straight setae of the prothoracic trochanterofemora resemble *Halictoxenos knereri*, whereas the meso- and metathoracic trochanterofemora have straight setae that resemble those of *Stylops melittae* (Pohl, 2000). The pro- and mesothoracic tarsi are adhesive structures (Pohl and Beutel, 2004) that would allow the larvae to stay on its mobile phoretic bee host, as well as to stay on its mother's host until departing onto a flower. The metathoracic leg is most likely used for movement and support of the larva's body, and does not appear to have a role in attachment by adhesion.

The dorsal and ventral sides of the abdomen were covered in spinulae, similar to the thorax. The pattern of spinula length is identical to that of *Stylops melittae* (Pohl, 2000). This pattern could be an autapomorphy throughout *Stylops* and may help the larva stay on the host in two possible ways. First, the spinulae may catch on the phoretic host's hairs if the first-instar loses contact with the host integument, enabling the larva to remain on the bee. Second, the

spinulae may catch on the rough surface of the pollen grains and help the larva travel with the pollen that is transferred to the corbiculae of the phoretic host. The spinulae may also be the initial way that a larva gets picked up, as these rigid structures may catch on the phoretic host's hairs when it is collecting pollen.

In most strepsipteran families, the caudal filaments are used to spring onto a host (Kirkpatrick, 1937; O'Conner, 1959; Young, 1987). However, *Stylops* has never been observed to jump, and is one of two genera (the other being *Halictoxenos*) that have dilated caudal filament tips (Pohl and Beutel, 2008). This suggests that the caudal filaments are instead additional attachment structures that help first-instars remain on their flying phoretic host (Ulrich, 1956; Borchert 1963).

The complex morphology of the first-instar larvae of *Stylops advarians*, and other *Stylops* species (Rohnstein, 1953; Borchert, 1963; Knauthe et al., 2016; Schneidereit, 1986; Pohl, 2000), as well as observations of larvae inside the digestive system of several bees (Linsley and MacSwain, 1957) and upon the surface of non-stylopized bees (Ulrich 1956), suggests *Stylops* first-instars can use two methods of travelling with a phoretic host to its nest. Until our study it was believed that travelling on the surface of the phoretic host is a behaviour that is less specialized than travelling within the phoretic host (Pohl and Beutel 2008). However, it seems the first-instars of *Stylops advarians* can use either method, perhaps depending on the precise location on the flower they occupy, as well as if the foraging bee is actively acquiring nectar or pollen. First-instar larvae of *Stylops* may accidently travel with a stylopized bee to that bee's nest. This would create a dead end for the *Stylops* larva, as stylopized bees are unable to produce any offspring, leaving the first-instar larvae without a host.

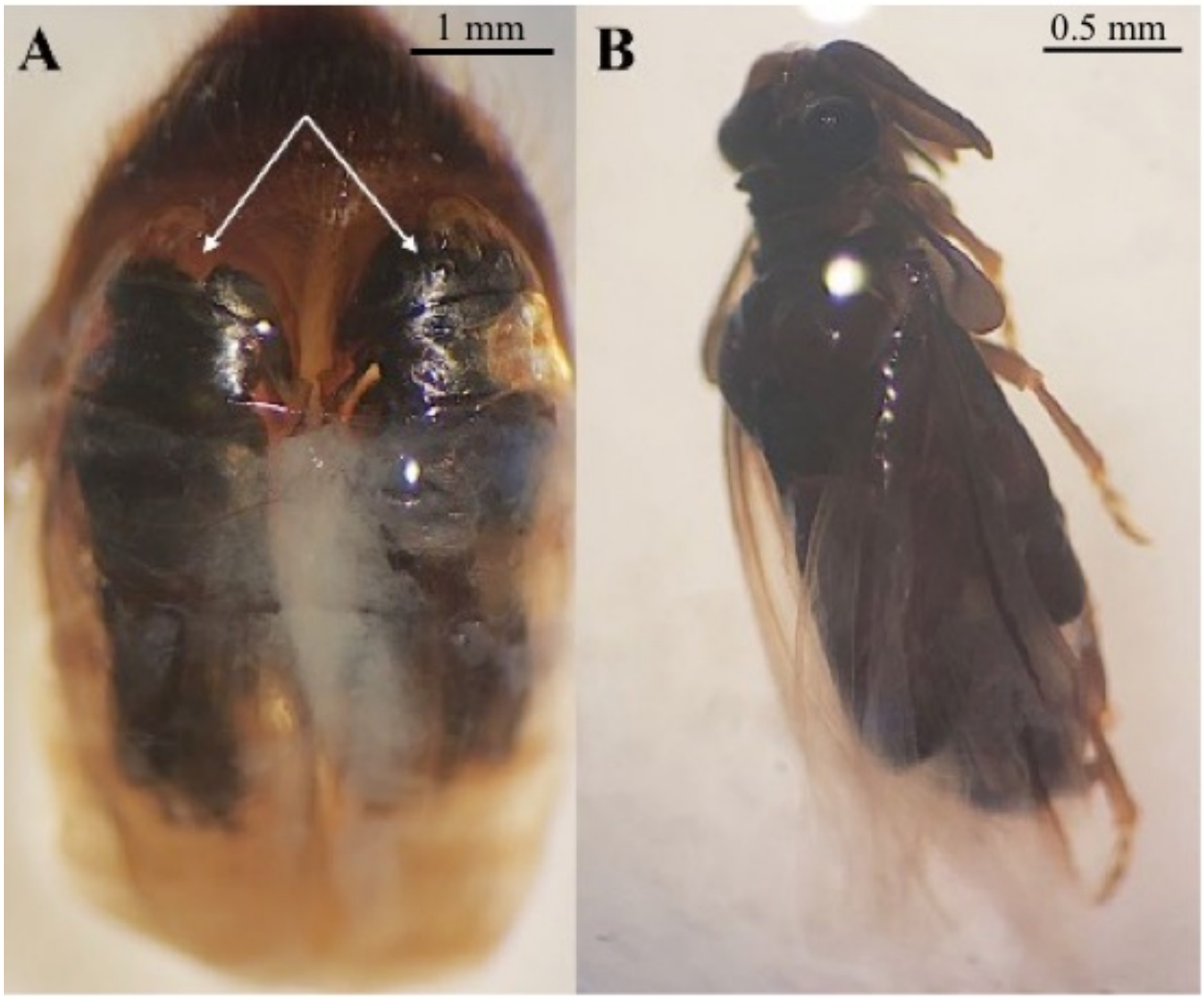
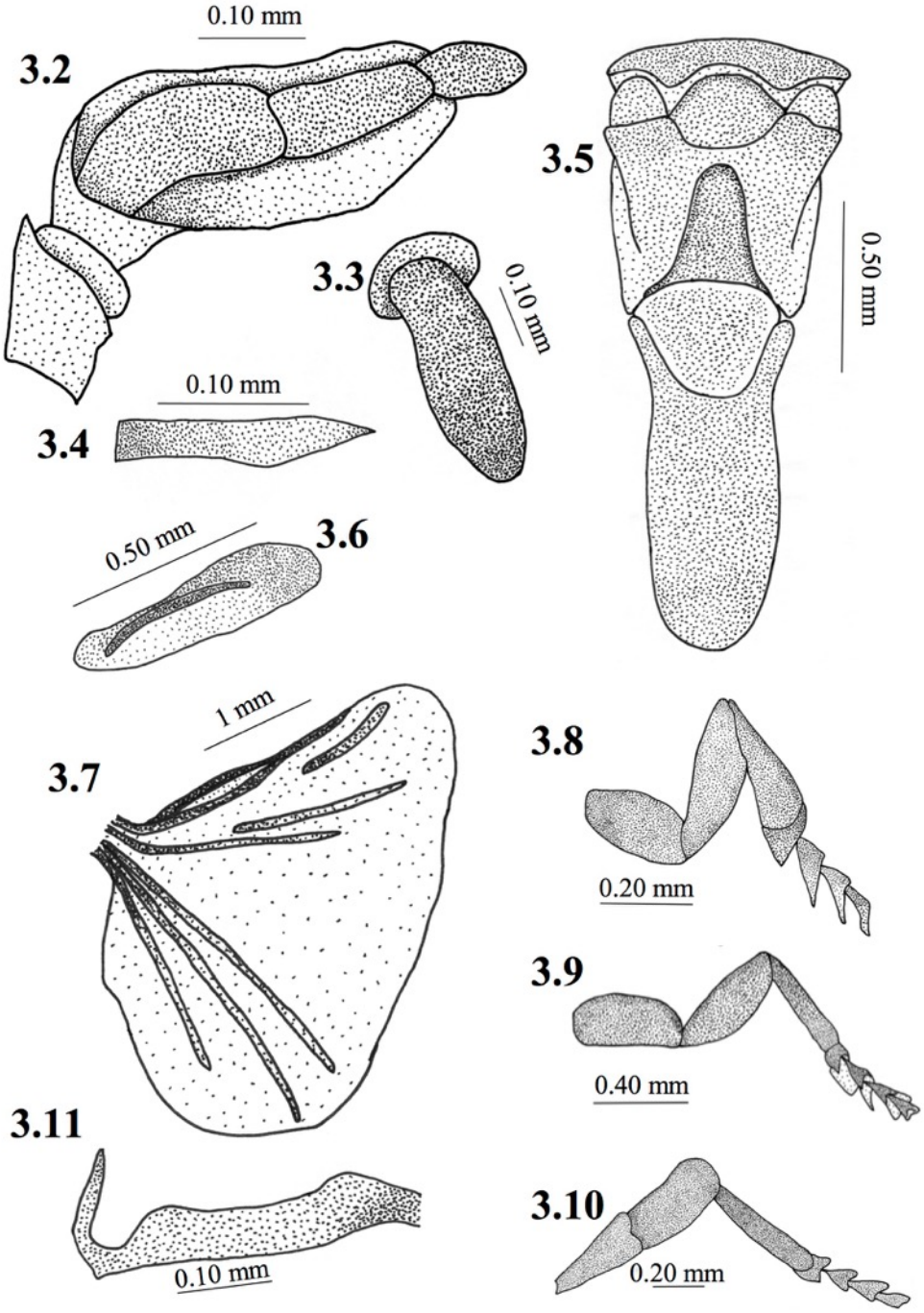


Figure 3.1. A: Infected abdomen of a bee of *Andrena milwaukeensis*, with the tergites removed, showing two puparia (arrows) of *Stylops advarians*. **B**: Adult male of *Stylops advarians* that has been removed from its puparium on the left side of the host's gaster.



Figures 3.2–3.11. *Stylops advarians*, adult male. 3.2: antenna, 3.3: maxillary palp, 3.4: mandible, 3.5: metathorax, 3.6: fore wing, 3.7: hind wing, 3.8: prothoracic leg, 3.9: mesothoracic leg, 3.10: metathoracic leg, 3.11: aedeagus.

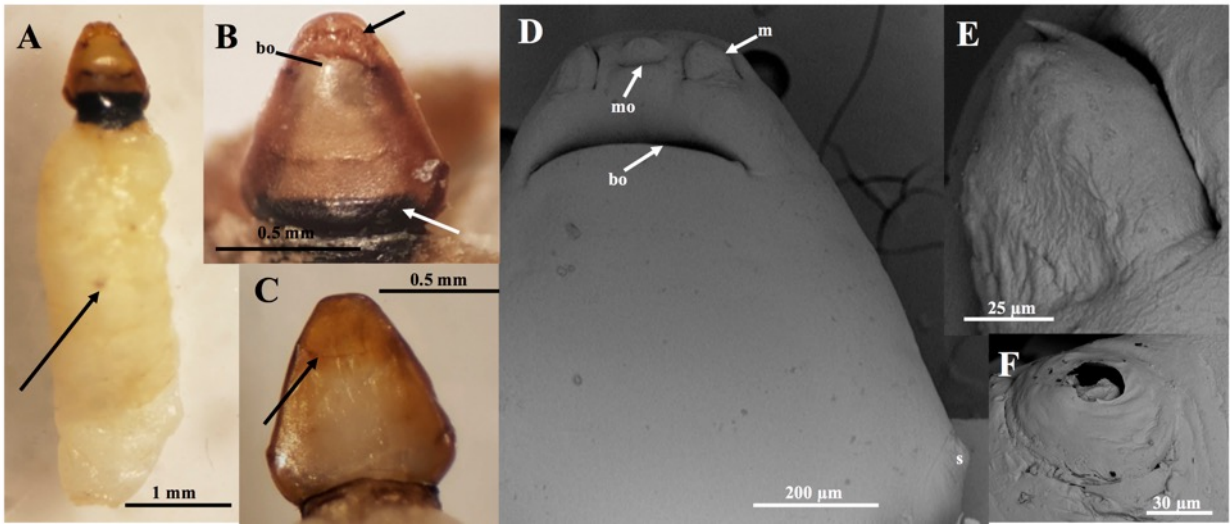


Figure 3.12. Adult female of *Stylops advarians*. **A**: Full body of the female removed from her *Andrena milwaukeensis* host. The abdomen is soft and larva-like. The brood canal is present and is more yellowish (arrow) than the rest of the abdomen, which is white. **B**: Dorsal view of the cephalothorax showing the brood opening, mandibles (black arrow), and the colouration pattern of abdominal segment I (white arrow). **C**: Ventral view of the cephalothorax showing the line where the head and thorax are fused (arrow). No fold lines are present. **D**: SEM-micrograph of the dorsal cephalothorax showing the mandibles, mouth opening, brood opening, and one spiracle. **E**: SEM-micrograph of one mandible, showing the mandible tooth. **F**: SEM-micrograph of one spiracle. — Abbreviations: bo – brood opening, m – mandible, mo – mouth opening, s – spiracle.

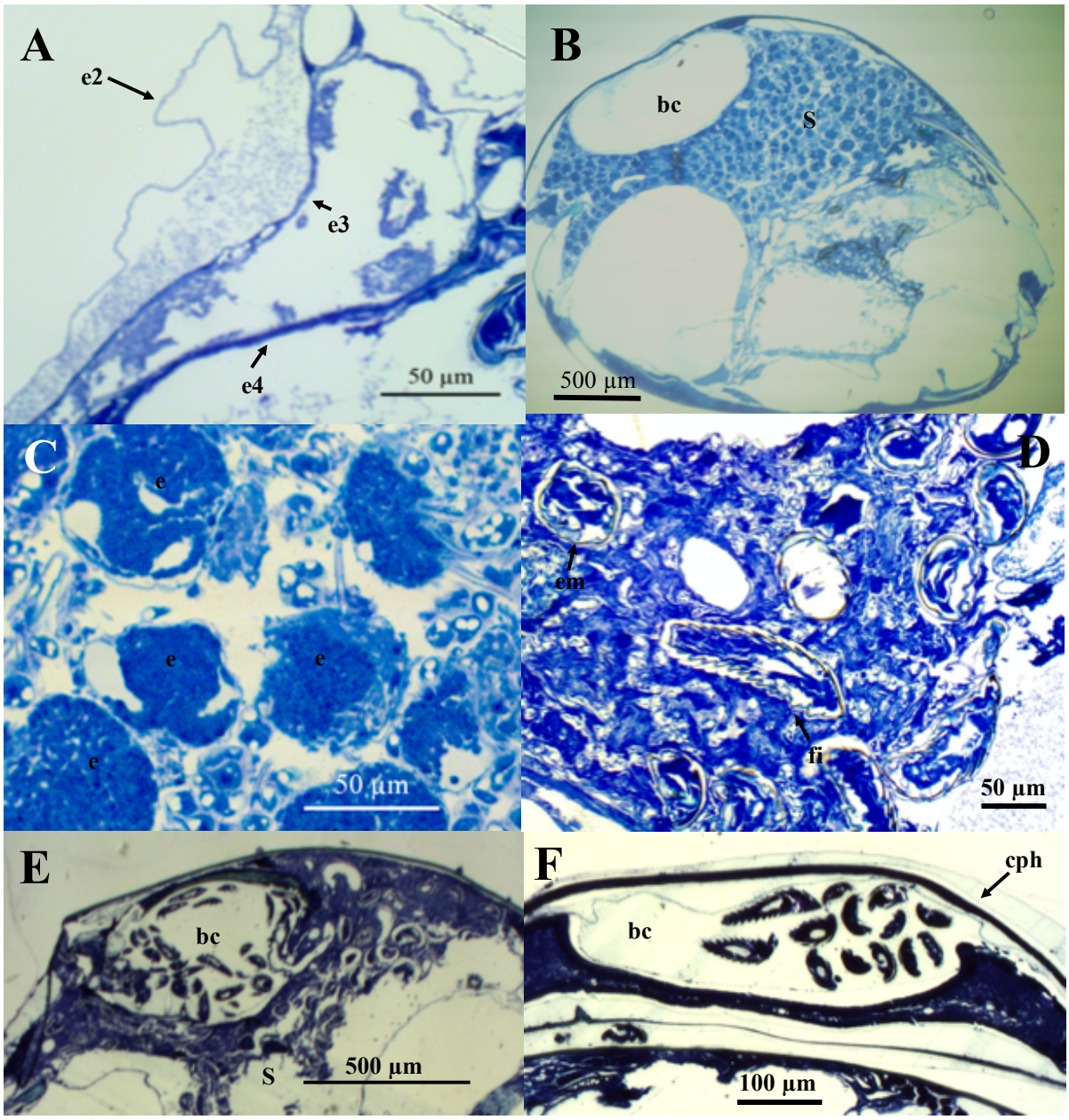


Figure 3.13. Abdomen of *Andrena milwaukeensis* stylopized by one *Stylops advarians* female. **A**: Cross section of the parasite's abdomen showing the three layers of exuvia that remain from its metamorphosis from the second, to third, to fourth-instar stages. **B**: Cross section of the bee's abdomen with a gravid female within. **C**: Eggs within female parasite. **D**: Cross section of gravid female showing asynchrony in progeny development, with a fully developed first-instar and an embryo in close proximity. **E**: Cross section of female parasite, showing several first-instar larvae within her brood canal. **F**: Cross section of the parasite's cephalothorax, showing several larvae within. — Abbreviations: bc – brood canal, cph – cephalothorax, e – egg, em – first-instar embryo, e2 – second-instar exuvium, e3 – third-instar exuvium, e4 – fourth-instar exuvium, fi – fully developed first-instar, S – Adult female *Stylops*. Fig. 3.13A,D – Specimen 7; Fig. 3.13B,C – Specimen 5; Fig. 3.13E,F – Specimen 8.

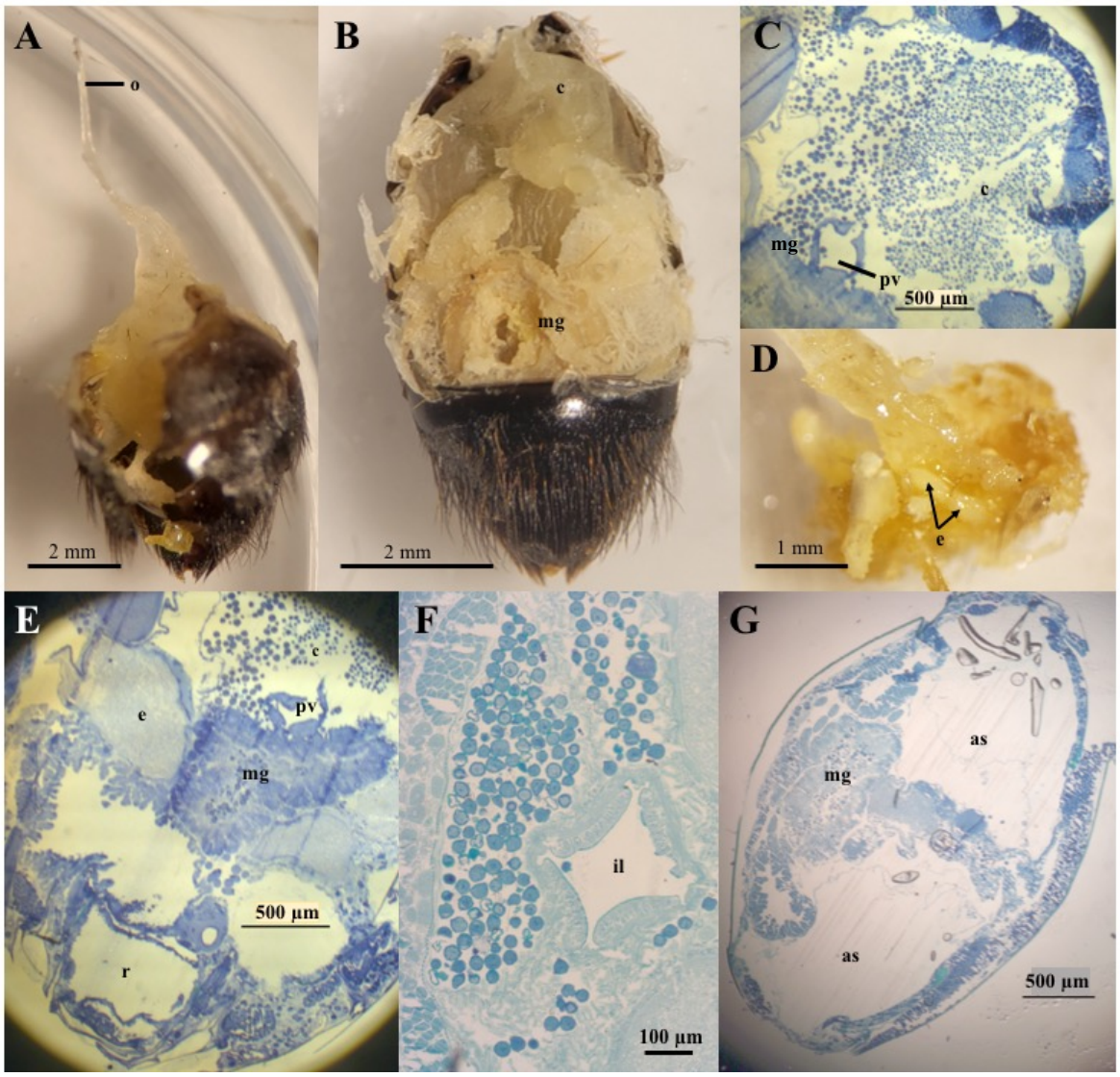


Figure 3.14. Photographs and light micrographs of the abdomen of several non-stylopized *Andrena milwaukeensis*. **A**: The bee's oesophagus leads from the thorax to the components of the abdominal digestive tract. **B**: The abdomen with several tergites removed to show the abdominal organs. The tergites covering the reproductive organs remain. **C**: Longitudinal section of the abdomen showing the crop (containing hundreds of pollen grains) leading to the proventriculus and anterior portion of midgut. **D**: Two eggs within the abdomen. **E**: Tangential section of the abdomen showing the centralized placement of several parts of the digestive tract, plus an egg. **F**: Cross section of the abdomen showing the ileum, which leads from the midgut to the pollen-filled rectum. **G**: Cross section of the abdomen showing the lateral placement of the two large air sacs within the abdomen. — Abbreviations: as – air sac, c – crop, e – egg, il – ileum, mg – midgut, o – oesophagus, pv – proventriculus, r – rectum. Fig. 3.14C,E – Specimen 3; Fig. 3.14F,G – Specimen 1.

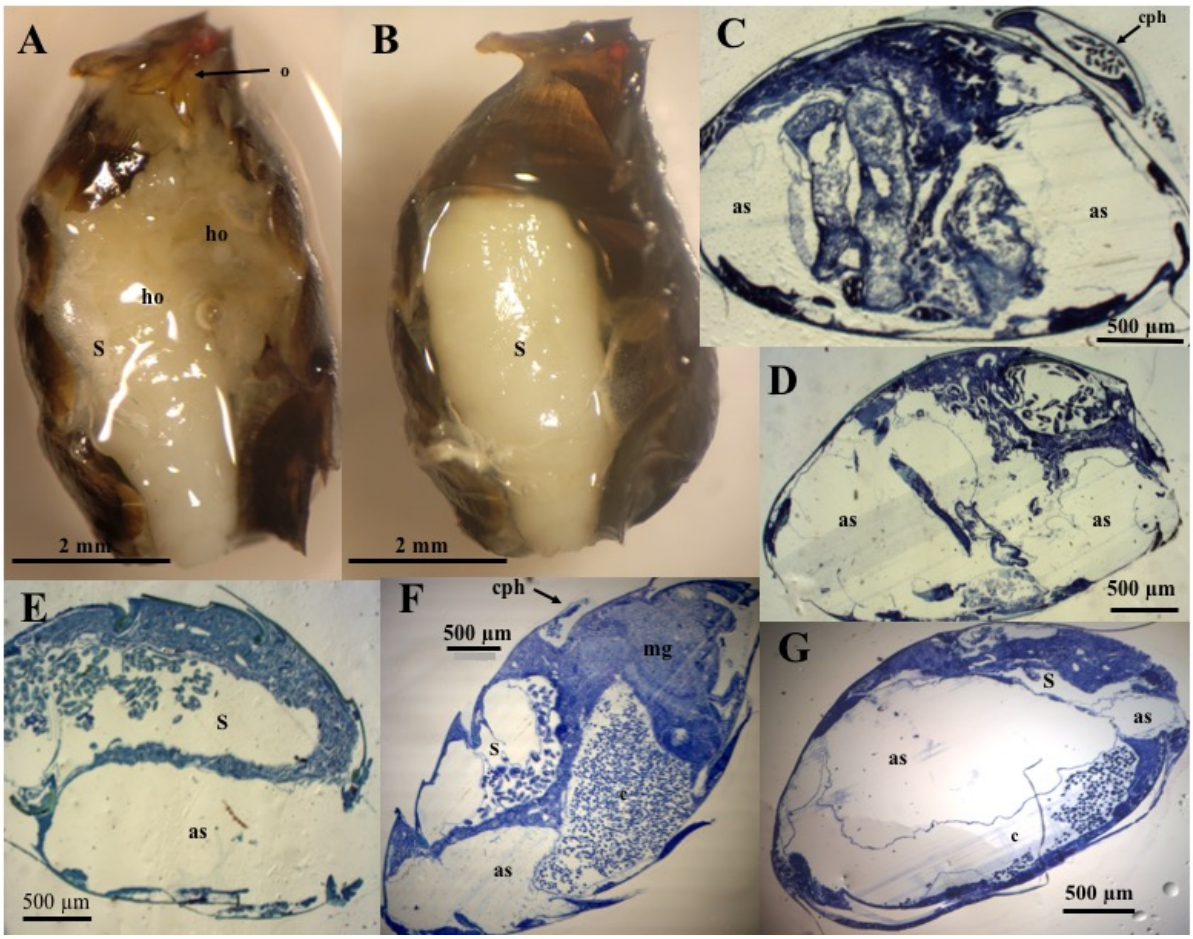


Figure 3.15. Abdomen of *Andrena milwaukeensis* infected with one adult female of *Stylops advarians*. **A**: Ventral view of host abdomen with the sternites removed, showing the female and the host's organs beside and below her. **B**: The same individual as **A**, but with the bee's organs removed. **C**: Cross section showing the placement of the air sacs near the posterior end of the host bee. The parasite's cephalothorax resides on the host's tergite. **D**: Cross section of host showing the female parasite's body above an air sac of the host. **E**: Tangential longitudinal section of the host showing the female parasite occupying one side of her host. **F**: Lateral section showing the female's abdomen occupying the upper portion of her host, with her body supported by an air sac and the crop. **G**: Cross section showing that the presence of the female strepsipteran causes the crop to expand away from her body. — Abbreviations: as – air sac, c – crop, cph – cephalothorax of female *Stylops*, ho – host's organs, mg – midgut, o – host's ovipositor, S – Female *Stylops*. Fig. 3.15C,D – Specimen 8; Fig. 3.15E – Specimen 10; Fig. 3.15F – Specimen 9; Fig. 3.15G – Specimen 6.

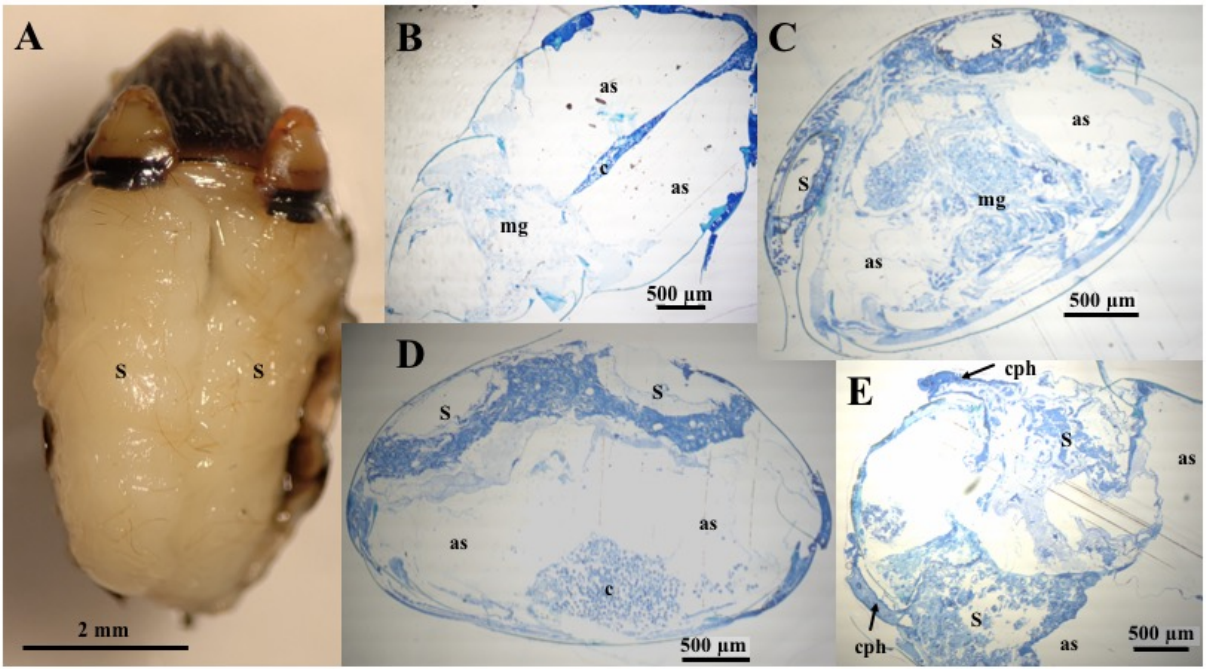


Figure 3.16. Abdomen of *Andrena milwaukeensis* infected with two adult females of *Stylops advarians*. **A**: Abdomen with the anterior tergites removed, showing the placement of the two adjacent females. **B**: Ventral-tangential section below the two strepsipterans, showing that the crop is heavily reduced in size. **C**: Cross section of the posterior end of a bee's abdomen showing the two strepsipterans on top of the host's tergites, but not yet localized within its body. **D**: Cross section of the middle of a bee's abdomen, showing the reduction of the crop when the two females are within their host's body. Both females are supported by one of the host's air sacs. **E**: Tangential section of the posterior end of a bee's abdomen, showing the two females localized at their transition from the external part of their host, where their cephalothoraces lay, to inside the host, where their abdomens reside. — Abbreviations: as – air sac, c – crop, cph – cephalothorax, mg – midgut, S – Female *Stylops*. Fig. 3.16B,E – Specimen 13; Fig. 3.16C,D – Specimen 12.

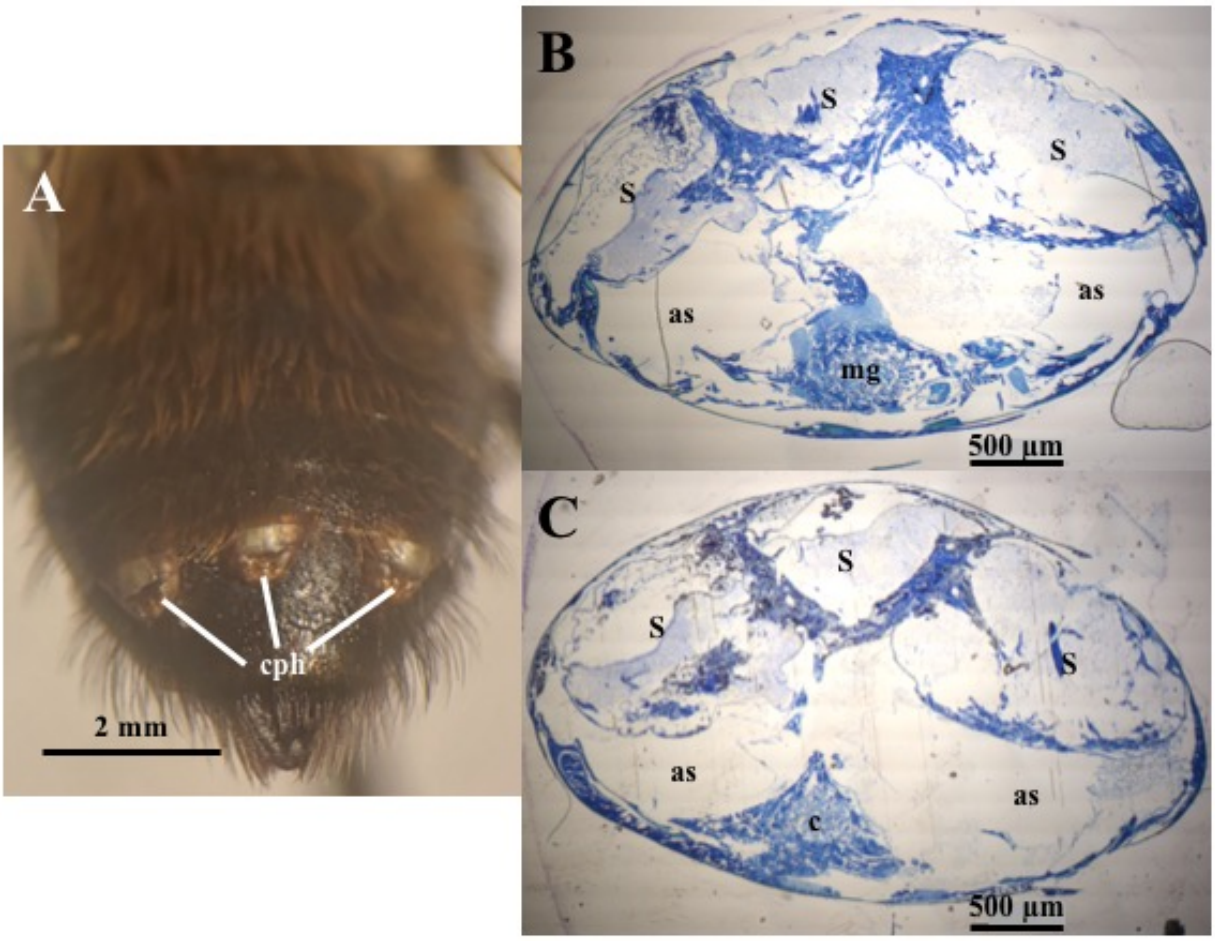


Figure 3.17. Abdomen of *Andrena milwaukeensis* infected with three adult females of *Stylops advarians*. **A**: Photograph of the abdomen showing the placement of the cephalothoraces of the three females. **B**: Cross section near the posterior end of a bee's abdomen, showing the reduced midgut, as well as the placement of the three females. The lateral females are each supported by one of the host's air sacs, whereas the central female is supported by the lateral females. **C**: Cross section near the middle of a bee's abdomen, showing the reduced crop. — Abbreviations: as – air sac, c – crop, cph – cephalothorax, mg – midgut, S – Female *Stylops*. Fig. 3.17B,C – Specimen 15.

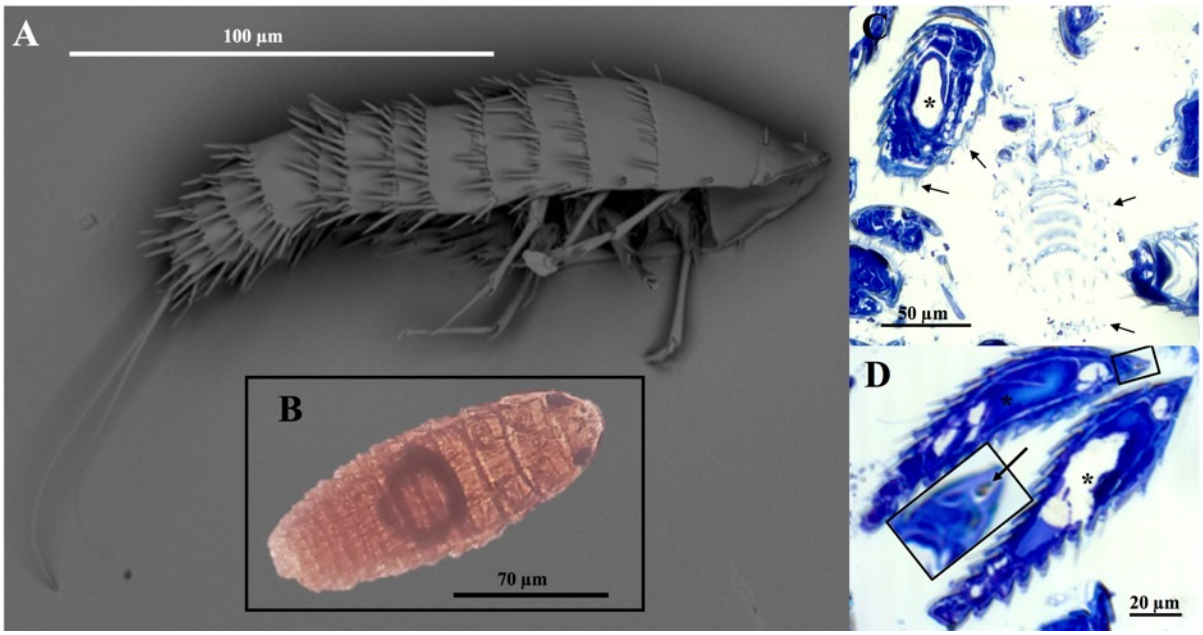


Figure 3.18. *Stylops advarians*, first-instar larvae. **A**: SEM micrograph, lateral view, **B**: Light micrograph of fresh specimen (mounted in water), dorsal view. An air bubble is evident ventrally. **C,D**: Histological semithin sections of numerous larvae in different orientations within the body cavity of their mother that protruded from abdomen of *Andrena milwaukeensis*, **C**: Dorsal view of near median-longitudinal section (at left) through larval abdomen with midgut (*). Larva (at right) sectioned tangential-longitudinally at its ventral surface through abdomen flexed between segments V and VI. Note sternal plates of thorax, basal portions of legs, abdominal segments of varying lengths, plus spinulae (arrows), **D**: Two larvae, lateral profile; note portion of digestive tract (*), mandibles (inset and arrow) in larva at left. Fig. 3.18C,D – Specimen 11.

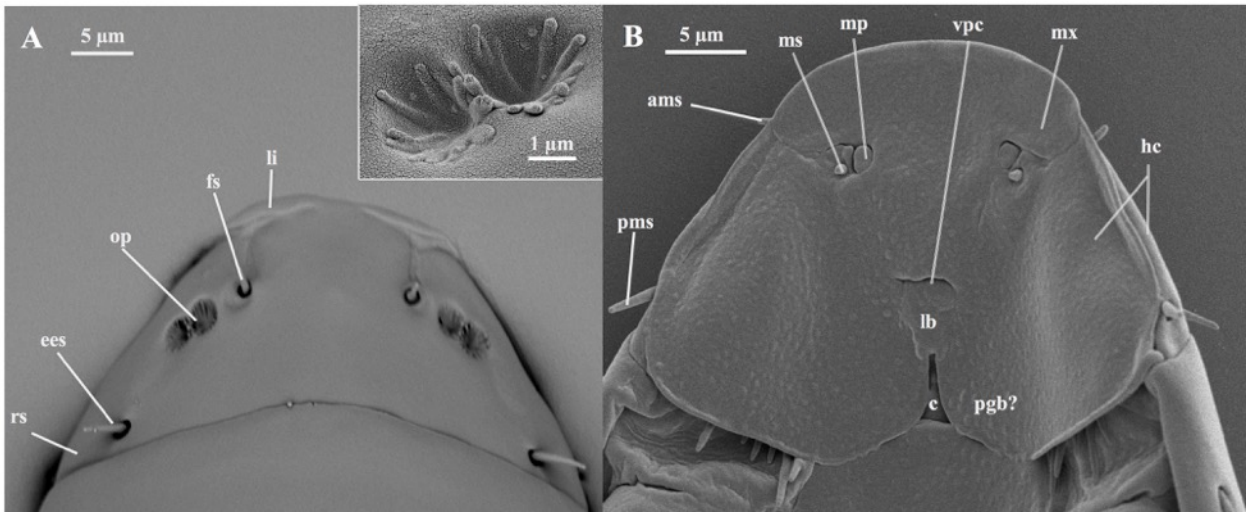


Figure 3.19. *Stylops advarians*, first-instar larva, head, SEM micrographs. **A**: dorsal view, **B**: ventral view. Inset of Figure 3.19A shows a higher magnification of the olfactory pit of the right side. — Abbreviations: ams – anterior marginal seta, c – cervix, ees – external eye seta, fs – frontal seta, hc – head capsule, lb – labium, li – lip-like structure, mp – maxillary palp, ms – maxillary seta, mx – maxilla, op – olfactory pit, pgb – postgenal bridge, pms – posterior marginal seta, rs – region of stemmata, vpc – ventral opening of preoral cavity.

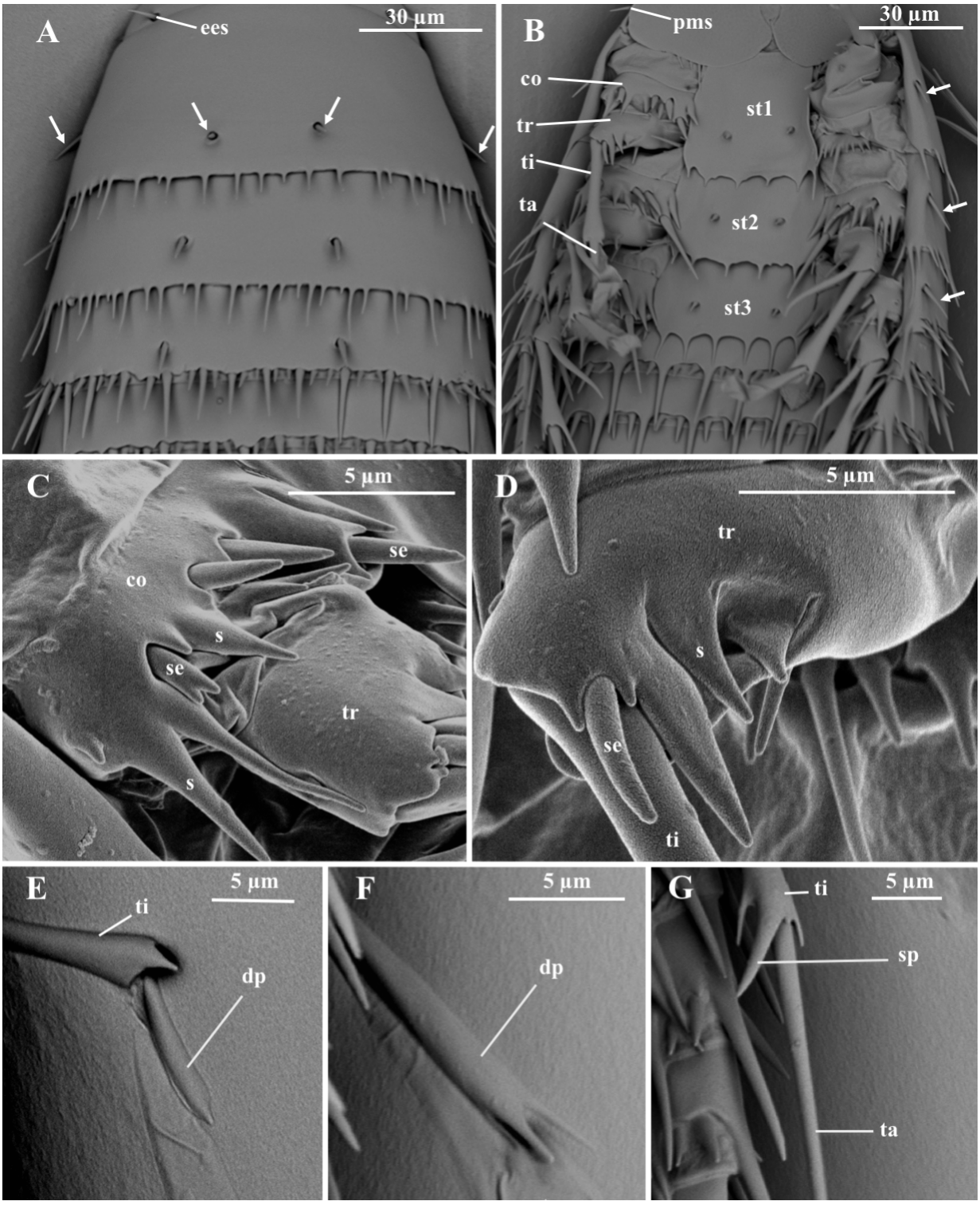


Figure 3.20. *Stylops advarians*, first-instar larva, SEM micrographs of thorax and legs. **A**: dorsal view; arrows indicate the four setae of the prothorax, **B**: ventral view; arrows denote three lateral setae on larva's left side, one seta per thoracic segment, **C**: prothoracic coxa, **D**: prothoracic trochanterofemur, **E**: prothoracic tarsus, **F**: mesothoracic tarsus, **G**: metathoracic tarsus. — Abbreviations: co – coxa, dp – dorsal plate of the tarsus, ees – external eye seta, pms – posterior marginal seta, s – spinula, se – seta, sp – tibial spur, st – sternal plate, ta – tarsus, ti – tibia, tr – trochanterofemur.

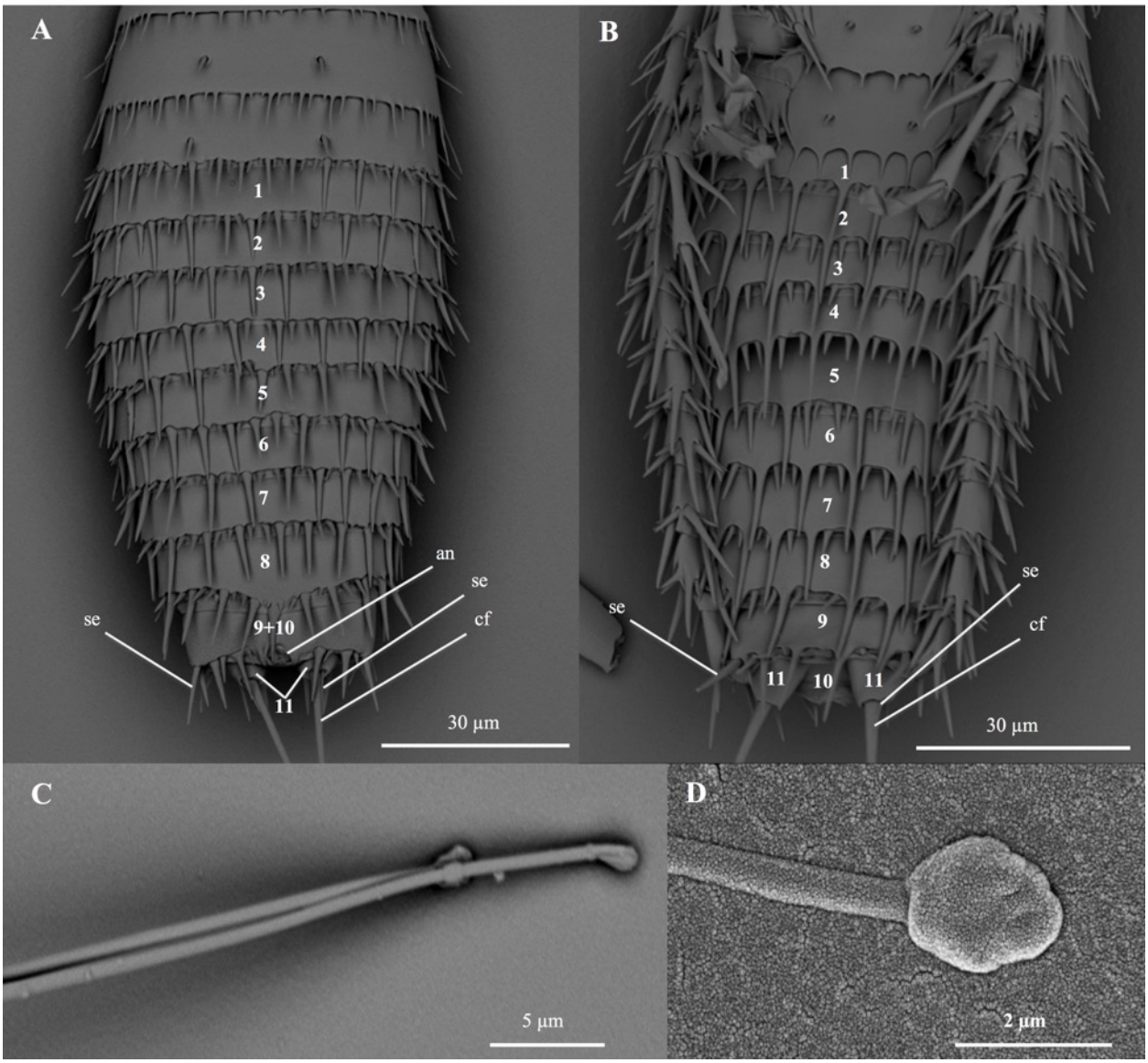


Figure 3.21. *Stylops advarians*, first-instar larva, abdomen, SEM micrographs. **A**: dorsal view, **B**: ventral view, **C**: caudal filament, **D**: caudal filament tip. — Abbreviations: an – anus, cf – caudal filament, se – seta.

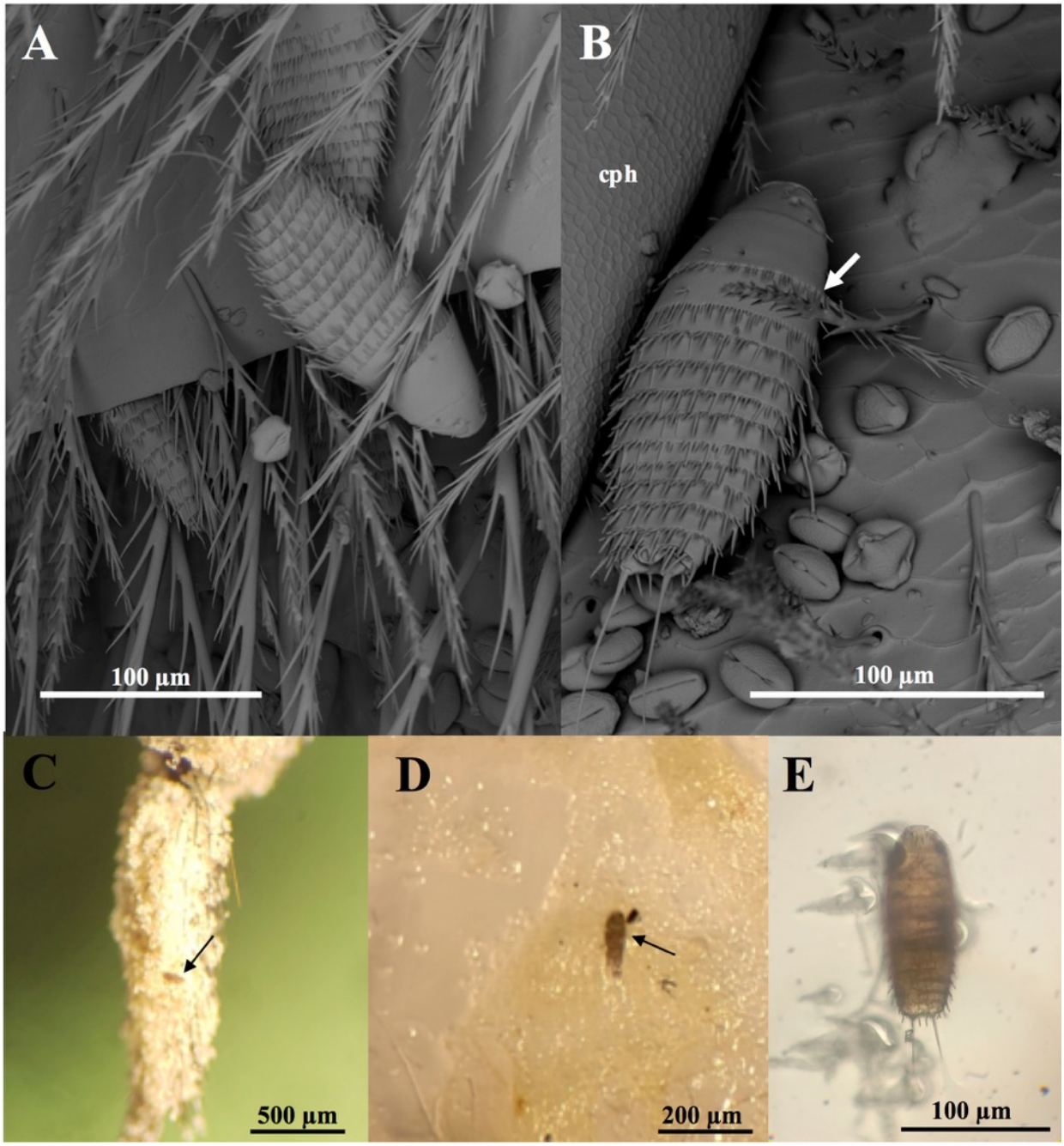


Figure 3.22. A,B *Stylops advarians*, recently emerged first-instar larvae on mother's host, *Andrena milwaukeensis*, SEM micrographs. **A** Three first-instar larvae entangled in the hairs or partially hidden below bee tergite, **B** A first-instar larva surrounded by small pollen grains on the surface of an abdominal tergite of mother's host. The larva has recently emerged from its mother (cph) and the spinulae of its prothoracic segment may be caught on the hairs of the bee (arrow), **C** First-instar larva (arrow) partially packed into the pollen pellet located on the hind basitarsus of a non-stylopized *Andrena milwaukeensis*. This individual is the same as that shown in Fig. 1B, **D** First-instar larva (arrow) detected amidst pollen within the honey sac of a non-stylopized *Andrena milwaukeensis*, **E** Increased magnification of the first-instar larva shown in Fig. 5D. — Abbreviations: cph – cephalothorax of female *S. advarians*.

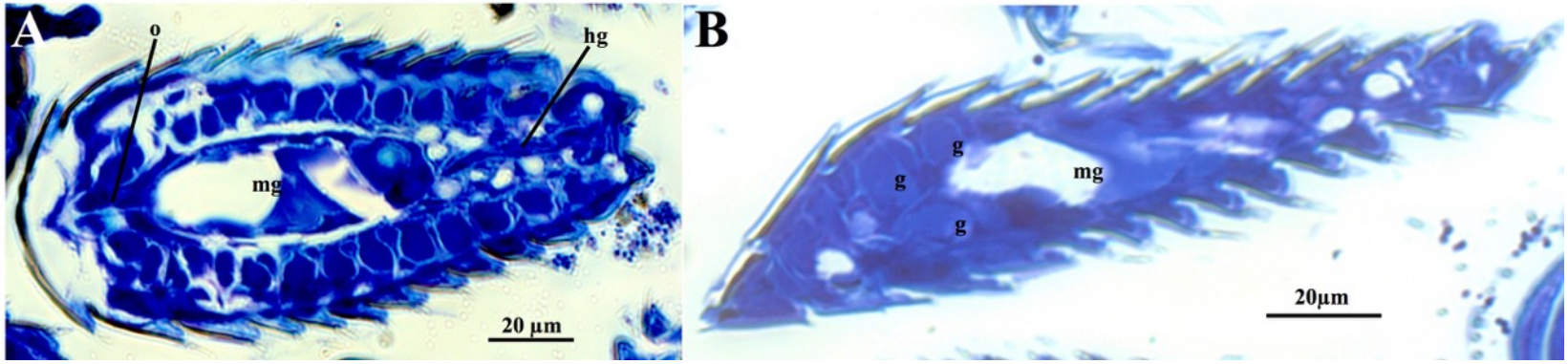


Figure 3.23. Histological sections of first-instar larvae of *Stylops advarians*. **A**: Near medial longitudinal section through larval body, showing part of the digestive tract throughout the body. **B**: Lateral view of tangential section through larval body showing the midgut and evidently three ganglia. — Abbreviations: g – ganglion, hg – hindgut, mg – midgut, o – oesophagus. Fig. 3.23A,B – Specimen 11.

CHAPTER 4: GENERAL DISCUSSION

4.1 Overview

This study involving *Stylops advarians* as a parasitic species of the ground-nesting bee species, *Andrena milwaukeensis*, has added support for several previous observations of *Stylops*, as well as several novel observations in regard to this parasite's life history and its impact on its host.

I have found support for the synchronization of male and female maturation (Hrabar et al., 2014), and that mating between the newly matured adults is aided by the natural advancement of the emergence time of stylopized female andrenids (Linsley and MacSwain, 1957; Straka et al., 2011). This manipulation of the emergence time of female andrenids, causing them to emerge earlier than normal, enables all strepsipterans to be in the environment at the same time, thus increasing each individual's chances of finding a mate.

Adult males of *S. advarians* were difficult to collect, due to their short life spans and extremely small size. Based on my observations of the timing of emergence of stylopized bees, and the initial emergence of the first-instar larvae, I expect that in the region of Cosmopolitan Park in Saskatoon, Saskatchewan, males of *S. advarians* emerge and mate around May 2 each year, but die, and hence disappear from the environment, shortly after copulation occurs. The rarity of finding intact puparia after May 2, and the presence of deceased males within these puparia, indicates that the males within intact puparia after May 2 were unable to emerge, not that these males normally emerge over an extended period of time.

Showing consistency over consecutive years, stylopized bees were present from around May 2 until around June 22. This 7-week period gives the female parasites time to mate, develop offspring, and release those offspring over several weeks, increasing her chances of having

multiple first-instar larvae find a host. First-instar larvae were initially observed emerging from their mother around May 23 each year, and then were found emerging for several weeks after that. This initial emergence suggests that, if development time of the embryos remains consistent each year, males and females emerge and mate around the same time every year. First-instar larvae were observed in various developmental stages within their mother's hemolymph. This asynchronous development of the first-instars is likely to take advantage of the many foraging trips that bees take over the season. A female releasing her offspring over a period of time in several locations, rather than all at once, may have a higher chance of having at least one offspring find a permanent, immature host in the nest of *A. milwaukeensis*.

The prevalence data were similar each year that collection of stylopized bees took place, and suggests that 22% (range 21–24%) of *A. milwaukeensis* in Saskatoon, Saskatchewan are stylopized by at least one female *S. advarians*. I was unable to determine the prevalence of bees infected with males, due to the difficulty of collecting stylopized bees that had evidence of a puparium. Without knowing the sex ratio of *S. advarians*, it is impossible to know the exact prevalence of the parasite, but it is surely above 22%.

Similarly, the mean intensity of parasite infection of 1.2 (range 1.17–1.24) and the parasite abundance of 0.27 (0.26–0.28) were remarkably consistent over the three consecutive years of this investigation, at this study site. Evidently this parasitic relationship of *S. advarians* on the bee host, *A. milwaukeensis*, is currently in balance.

One bee with two puparia was collected by using a sweep net. This bee had its abdomen separated from the thorax, and the tergites removed to reveal the entirety of the two puparia. One puparium was removed from the abdomen and the male was extracted from the casing. This male was used to provide the first morphological description of an adult male of *S. advarians*, a

species which had previously only been known from the neotenic female (Pierce, 1909). However, the DNA extracted from a single hind leg of this male was insufficient to identify this specimen to species. Accordingly, the entire male from the second puparium was utilized successfully by molecular investigation, to confirm the species identity as *S. advarians*.

The adult female of *S. advarians* greatly resembles the adult females of other *Stylops* species. The only identifiable characteristics, the mandibles, colouration pattern, and spiracles, are found on the cephalothorax, and these features cannot be used to accurately identify to species. Except for its first segment, the entire abdomen is soft and larva-like, and is full of the female's eggs, or developing larvae once fertilization has occurred. A discoloured region is present from abdominal segments II–VI, which signifies where the brood canal is located on the female. The brood canal is used by fully developed first-instar larvae as a pathway to travel outside of their mother, through her cephalothorax, allowing their ultimate escape at the brood opening.

The adult females of *S. advarians* have a substantial impact on their host's anatomy. I found that the presence of at least one adult female of *S. advarians* led to the absence of mature ovaries in female *A. milwaukeensis*. This discovery suggests that at least 22% of this *A. milwaukeensis* population are unable to reproduce, due to being infected with *S. advarians*. Other findings on the impact of the female parasite on her host are the shift of organs to the side opposite of the parasite, as well as limiting the space that the host's crop has to expand. I found that the organs closer to the posterior end of the bee were relatively unaffected because the female parasite's body did not occupy this posterior region of the host. When two or three female parasites are present, the crop is further limited in how much it can expand. Like with one female, however, the organs in the posterior region of the bee's abdomen remain relatively

unaffected. In instances where a bee was infected with one or two female parasites, these parasites were found laterally in the abdomen. When a third female parasite was present, it was found in the centre of the host abdomen, between the two laterally-positioned parasites. Females that occupied the lateral area of the host's abdomen were found to be supported by one of the host's air sacs. If a third female was present, she was supported by the abdomens of the other two female parasites.

My findings on the structure of the first-instar larva of *S. advarians* are similar to those from other *Stylops* species (*Stylops melittae* in Pohl, 2000; *Stylops ovinae* in Knauthe et al., 2016). One important observation was the presence of a first-instar larva partially packed into the pollen on the hind leg of a non-stylopized *A. milwaukeensis*. This occurrence demonstrates that these first-instars are able to travel on the surface of an adult bee host to its nest, and may be well adapted to do so. A first-instar larva was also found within the crop of a non-stylopized *A. milwaukeensis*. Linsley and MacSwain (1957) also reported finding first-instar larvae within the crop of an andrenid bee, but they were unable to find phoronts on the surface of the andrenids. It has been suggested that travelling on the surface of an adult bee host is a less specialized form of phoresy compared to travelling within the crop (Pohl and Beutel, 2008), but my findings suggest that the same species of *Stylops* may have the ability to travel on the surface of, or within, an adult bee, and be transported to that bee's nest.

4.2 Future Research

Strepsiptera is a fascinating order that is unfortunately understudied. Much more work is not only required to better understand the life history of *Stylops* species, including the impact on their hosts, but also every other strepsipteran genus. An important step is going to be making

sure that our understanding of the strepsipteran phylogeny is correct. Many descriptions of these species were made before molecular techniques were established (Bohart, 1941; Pierce, 1909, 1911, 1914, 1918; Perkins, 1918); thus, it is unclear how many species there are, and how these species are related to one another. Recently, 110 *Stylops* species were examined using cytochrome c oxidase subunit I, and it was found that many of the species that had been described were misidentified (Jůzova et al., 2015; Straka et al., 2015). This misidentification is due to the cryptic nature of Strepsiptera (Hayward et al., 2011; Kathirithamby et al., 2015). Work like that done by Straka et al. (2015) which has reduced the accepted number of *Stylops* species to 67, needs to be continued for the other genera to determine the diversity of these unique parasites. A broader study is still required to provide evidence for the ordinal status of Strepsiptera. Strepsipterans are so distinct from their sister group, Coleoptera, that it seems clear that they should belong to their own order, but more molecular work should be done to confirm this hypothesis.

Further investigation into the host-parasite relationships and life history of Canadian Strepsiptera, the numbers of which were obtained by Straka (2019), is also required, as there has been very little study on these species. Hrabar et al. (2014) recently found several important life history traits of *Xenos peckii* that had not been previously documented. Several strepsipteran species were described and studied in the early 20th century (Pierce, 1909; MacKay, 1938), but little is known about their biology, ecology, life history, and host impact. The directions of study mentioned in the proceeding paragraphs could easily be completed on the five families of Canadian Strepsiptera to further our knowledge of this unique group of insects. Additionally, comparisons can be done on the impact of *Stylops advarians* on other *A. milwaukeensis* populations, including those in New York and Maine (Jůzova et al., 2015) and British Columbia

(Pierce 1909), as well as how *S. advarians* impacts its other hosts, *A. frigida*, *A. mandibularis*, and *A. vicinoides*.

Adult males of *Stylops* often go uncollected, as they are very difficult to find. The acquisition of live adult males from more species will likely help with understanding their reproductive behaviours, mate-seeking behaviours, as well as anatomy and morphology. Very little information is available on the morphology of most species of adult male *Stylops*, which should be addressed by collecting and describing males. If more adult male specimens were available to me, I would have liked to examine them using scanning electron microscopy. These images could reveal tiny features of the adult male impossible to see using a dissecting microscope.

Several aspects of the life history of *Stylops* are still unknown. It is currently unclear which host stage is parasitized by first-instar larvae of *Stylops*. All strepsipterans infect an immature stage, but this is typically the larval or nymphal stage (Kathirithamby, 1989, 2009). *Stylops* is thought to penetrate the egg of its permanent *Andrena* host (Linsley and MacSwain, 1957; Knauthe et al., 2016), but there are no direct observations of this activity. I suggest that based on the mass provisioning behaviour of the host andrenid bees, along with observations of Linsley and MacSwain (1957) and Knauthe et al. (2016), first-instar larvae infect the egg stage. Other unknowns are how the first-instar larvae attach onto the surface of the adult bee host that transports them to the bee's nest. It is unclear how important the spinulae on the dorsal and ventral surfaces of first-instar larvae of *Stylops* are for getting onto an adult bee host, for staying on the host's surface, or both. First-instar larvae of *S. advarians* possess many spinulae on both their dorsal and ventral surfaces, and appear well adapted to attach and travel on their hairy host, the adult bees of *A. milwaukeensis*. I suggest that these spinulae are important for accessing hairy

hosts, with evidence of first-instar larvae that infect hosts with little hair having few spinulae, or only have spinulae on their ventral side, whereas first-instar larvae with hairy hosts have many spinulae (Pohl, 2000; Fig. 11, 12; Pl. 22–31).

For the first-instars that travel on the surface of an adult bee, it is suggested that the flattened pro-and mesothoracic tarsi are used for adhesion (Beutel and Gorb, 2001; Pohl and Beutel, 2004). Further investigation of the caudal filaments is required to determine if the caudal filaments are adhesive structures. Observations of the first-instars on inverted surfaces may be able to answer the question about the caudal filaments, as well as confirm the hypothesis about the adhesiveness of the tarsi.

It is currently unclear how strepsipteran first-instar larvae respire. No spiracles were observed in my examination of the bodies of many first-instar larvae of *Stylops advarians*, and have not been found in other studies (Rohnstein 1953; Pohl, 2000; Knauthe et al., 2016). The first-instars are known to have a tracheal system (Rohnstein, 1953), but it is unclear how the air from the external environment passes into their tracheae. These minute larvae may be able to use diffusion to acquire oxygen when they are in the external environment. Perhaps *Stylops* first-instar larvae, which can be found alive in the crop of an adult bee host (Linsley and MacSwain 1957), use air bubbles within the host's collected nectar to obtain oxygen. The concave body of the larvae might allow for an air bubble to be trapped and used to gather air (Fig 3.18B), though this concavity is likely an artifact from the drying process.

The morphology of the second, third, and fourth-instars of *Stylops* is also unknown. This lack of knowledge is due to the difficulty in finding and excavating *Andrena* nests. It will be important to understand how these later instars survive inside their host, and to note if the host-derived epidermal bag used in immune evasion is the same as that found in Elenchidae and

Xenidae (Kathirithamby et al., 2003; Manfredini et al., 2007). Manfredini et al. (2007) suggest that the first-instars further avoid their host's immune system by using surface factors, which inhibit the host from recognizing the parasite as non-self (Schmidt et al., 2001).

Bee health is an important area of study, with many groups around the world investigating the impact that parasites, pathogens, and invasive plant and insect species have on honeybee and native bee species (Vanderplanck et al., 2019). A recent study on the abundance of *Varroa* mites parasitizing *Apis mellifera* found that these parasites are much more abundant in the examined colonies than previously thought (Traynor et al., 2016). If well-studied honeybee colonies can have a surprise increase in abundance of harmful parasites, then the same is likely true for other understudied important bee pollinator species. A continued lack of knowledge on native bee species could be extremely detrimental because there will not be enough measures to protect them from disease and parasites.

A better understanding of vectors of disease and parasites is required to decrease the loss of bees to pathogens and parasites. Perhaps an important step will be the management of the landscape, as well as management of the number of flowers within an environment. Cohen (2018) found that the abundance of the parasitoid fly, *Apocephalus borealis*, increased when more shrubs and trees were around the garden. However, Cohen (2018) also found that increasing the abundance of floral resources in urban gardens increased the number of bumblebees and honeybees infected with parasites and pathogens.

More study is required on the host-parasite relationship between native bees and their parasites to learn the impact that parasites, including the strepsipterans which infect four families of bees (Kathirithamby, 2018), have on these essential pollinators. More study is also required to

determine different management strategies that will allow important bee pollinators to reduce their risk for disease and parasitization.

The host-parasite relationship between *A. milwaukeensis* and *S. advarians* in Cosmopolitan Park in Saskatoon, Saskatchewan is currently in balance. However, their environment along the Meewasin Trail is often being modified by humans, so these changes may put this population at risk of losing the resources it needs, which may cause an outbreak of bees infected with *S. advarians*. Even if the prevalence of the parasite remains the same, pesticide use may affect development speed, body mass, and bee longevity (Anderson and Harmon-Threatt, 2019). If an outbreak of *S. advarians* occurred when many bees were struggling with development and longevity, then the population of *A. milwaukeensis* may not be able to recover.

Ultimately, much more study is needed on *S. advarians* and its host *A. milwaukeensis*, as well as many other *Stylops* and strepsipteran species. Until these studies are done, we cannot begin to understand the impact that species of Stylopidae have on their important bee pollinator hosts.

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APPENDIX

The following tables provide the dates when collection of *Andrena milwaukeensis* took place, beginning in 2016 and finishing in 2018. Furthermore, the sex of the captured bee, the number of bees collected per day, and whether or not each bee was stylopized, are indicated below. Under the stylopized column, an 'N' means that the bee was not stylopized, and a 'Y' means that the bee was stylopized; the number in brackets indicates how many female *Stylops advarians* were infecting each stylopized bee.

Table A.1. 2016

Date Collected	Sex of Bee	Bee Number Assigned Per Day	Stylopized?
04-May-16	F	1	N
05-May-16	F	1	N
06-May-16	F	1	N
06-May-16	F	2	N
06-May-16	F	3	N
16-May-16	F	1	N
16-May-16	F	2	N
16-May-16	F	3	N
17-May-16	F	1	N
18-May-16	F	1	N
18-May-16	F	2	N
18-May-16	F	3	N
18-May-16	F	4	N
18-May-16	F	5	N
18-May-16	F	6	N
19-May-16	F	1	N
19-May-16	F	2	N
19-May-16	F	3	N
19-May-16	F	4	N
19-May-16	F	5	N
19-May-16	F	6	N
20-May-16	F	1	N
20-May-16	F	2	N
20-May-16	F	3	Y (1)
20-May-16	F	4	N
25-May-16	F	1	N

25-May-16	F	2	N
25-May-16	F	3	N
25-May-16	F	4	N
25-May-16	F	5	Y (1)
25-May-16	F	6	N
25-May-16	F	7	N
25-May-16	F	8	N
27-May-16	F	1	Y (1)
27-May-16	F	2	N
27-May-16	F	3	N
27-May-16	F	4	N
27-May-16	F	5	N
27-May-16	F	6	N
27-May-16	F	7	N
27-May-16	F	8	N
30-May-16	F	1	Y (1)
30-May-16	F	2	Y (1)
30-May-16	F	3	N
30-May-16	F	4	N
30-May-16	F	5	N
30-May-16	F	6	N
30-May-16	F	7	N
30-May-16	F	8	N
30-May-16	F	9	N
30-May-16	F	10	N
30-May-16	F	11	N
30-May-16	F	12	N
31-May-16	F	1	N
31-May-16	F	2	N
31-May-16	F	3	Y (1)
31-May-16	F	4	Y (1)
31-May-16	F	5	Y (2)
31-May-16	F	6	N
31-May-16	F	7	N
01-Jun-16	F	1	N
01-Jun-16	F	2	N
01-Jun-16	F	3	Y (1)
02-Jun-16	F	1	N
02-Jun-16	F	2	N
02-Jun-16	F	3	N
02-Jun-16	F	5	Y (1)
02-Jun-16	F	6	Y (1)
02-Jun-16	F	7	Y (1)
02-Jun-16	F	8	N
02-Jun-16	F	9	N

03-Jun-16	F	1	N
03-Jun-16	F	2	N
03-Jun-16	F	3	N
03-Jun-16	F	4	N
03-Jun-16	F	5	N
03-Jun-16	F	6	Y (1)
03-Jun-16	F	7	Y (1)
03-Jun-16	F	8	Y (1)
03-Jun-16	F	9	Y (1)
03-Jun-16	F	11	Y (1)
03-Jun-16	F	12	N
03-Jun-16	F	13	N
06-Jun-16	F	1	N
06-Jun-16	F	2	N
06-Jun-16	F	3	Y (1)
07-Jun-16	F	1	N
07-Jun-16	F	2	N
07-Jun-16	F	3	Y (1)
07-Jun-16	F	4	Y (1)
08-Jun-16	F	1	Y (1)
08-Jun-16	F	2	Y (3)
08-Jun-16	F	3	N
08-Jun-16	F	4	N
08-Jun-16	F	5	N
08-Jun-16	F	6	Y (1)
09-Jun-16	F	1	Y (1)
09-Jun-16	F	2	N
09-Jun-16	F	3	N
09-Jun-16	F	4	Y (1)
10-Jun-16	F	1	N
10-Jun-16	F	2	Y (3)
10-Jun-16	F	3	N
10-Jun-16	F	4	N
10-Jun-16	F	5	N
10-Jun-16	F	6	N
10-Jun-16	F	7	N
13-Jun-16	F	1	Y (1)
13-Jun-16	F	2	Y (1)
13-Jun-16	F	3	Y (1)
13-Jun-16	F	4	N
13-Jun-16	F	5	N
13-Jun-16	F	6	N
13-Jun-16	F	7	N
13-Jun-16	F	8	N
15-Jun-16	F	1	N

15-Jun-16	F	2	Y (1)
15-Jun-16	F	3	N
15-Jun-16	F	5	N
17-Jun-16	F	1	N
17-Jun-16	F	2	N
17-Jun-16	F	3	N
17-Jun-16	F	4	N
17-Jun-16	F	5	N
17-Jun-16	F	6	N
20-Jun-16	F	1	N
20-Jun-16	F	2	N

Table A.2. 2017

Date Collected	**Sex of Bee**	**Number Collected Per Day**	**Stylopized?**
03-May-17	F	1	Y (1)
03-May-17	F	2	Y (1)
03-May-17	F	3	Y (1)
03-May-17	F	4	Y (1)
04-May-17	F	1	Y (1)
04-May-17	F	2	Y (1)
04-May-17	F	3	N
04-May-17	F	4	N
05-May-17	F	1	Y (2)
15-May-17	F	1	Y (2)
15-May-17	F	2	N
15-May-17	F	3	N
15-May-17	F	4	N
15-May-17	F	5	N
16-May-17	F	1	N
16-May-17	F	2	N
16-May-17	F	3	N
16-May-17	F	4	N
16-May-17	F	5	N
16-May-17	F	6	N
18-May-17	F	1	N
18-May-17	F	2	Y (3)
18-May-17	F	3	N
18-May-17	F	4	N
18-May-17	F	5	N
18-May-17	F	6	N
18-May-17	F	7	N
18-May-17	F	8	N
19-May-17	F	1	N

19-May-17	F	2	N
19-May-17	F	3	N
23-May-17	F	1	N
23-May-17	F	2	N
23-May-17	F	3	N
23-May-17	F	4	N
23-May-17	F	5	N
23-May-17	F	6	N
23-May-17	F	7	N
23-May-17	F	8	N
23-May-17	F	9	N
23-May-17	F	10	N
24-May-17	F	1	N
24-May-17	F	2	N
24-May-17	F	3	Y (1)
24-May-17	F	4	N
24-May-17	F	5	N
24-May-17	F	6	N
24-May-17	F	7	N
24-May-17	F	8	N
24-May-17	F	9	N
25-May-17	F	1	N
25-May-17	F	2	N
25-May-17	F	3	N
25-May-17	F	4	N
25-May-17	F	5	N
25-May-17	F	6	N
25-May-17	F	7	N
29-May-17	F	1	Y (2)
29-May-17	F	2	N
29-May-17	F	3	N
30-May-17	F	1	N
30-May-17	F	2	N
30-May-17	F	3	N
30-May-17	F	4	N
31-May-17	F	1	Y (1)
31-May-17	F	2	N
31-May-17	F	3	N
31-May-17	F	4	N
01-Jun-17	F	1	N
01-Jun-17	F	2	N
03-Jun-17	F	1	N
03-Jun-17	F	2	N
03-Jun-17	F	3	N
05-Jun-17	F	1	N

05-Jun-17	F	2	Y (1)
05-Jun-17	F	3	Y (1)
05-Jun-17	F	4	Y (1)
05-Jun-17	F	5	Y (1)
05-Jun-17	F	6	N
05-Jun-17	F	7	N
06-Jun-17	F	1	N
06-Jun-17	F	2	N
06-Jun-17	F	3	N
06-Jun-17	F	4	N
07-Jun-17	F	1	N
07-Jun-17	F	2	Y (1)
07-Jun-17	F	3	N
07-Jun-17	F	4	N
07-Jun-17	F	5	N
07-Jun-17	F	6	N
07-Jun-17	F	7	N
07-Jun-17	F	8	N
08-Jun-17	F	1	Y (1)
08-Jun-17	F	2	N
08-Jun-17	F	3	N
08-Jun-17	F	4	N
10-Jun-17	F	1	Y (1)
10-Jun-17	F	2	N
10-Jun-17	F	3	N
10-Jun-17	F	4	N
10-Jun-17	F	5	N
12-Jun-17	F	1	Y (2)
12-Jun-17	F	2	N
12-Jun-17	F	3	Y (1)
12-Jun-17	F	4	Y (1)
12-Jun-17	F	5	N
12-Jun-17	F	6	N
13-Jun-17	F	1	Y (1)
13-Jun-17	F	2	N
13-Jun-17	F	3	N
13-Jun-17	F	4	N
13-Jun-17	F	5	N
13-Jun-17	F	6	N
13-Jun-17	F	7	N
16-Jun-17	F	1	Y (1)
16-Jun-17	F	2	N
16-Jun-17	F	3	N
16-Jun-17	F	4	Y (1)
16-Jun-17	F	5	N

16-Jun-17	F	6	N
16-Jun-17	F	7	N
16-Jun-17	F	8	N
16-Jun-17	F	9	N
19-Jun-17	F	1	N
19-Jun-17	F	2	N
19-Jun-17	F	3	Y (1)
19-Jun-17	F	4	Y (1)
19-Jun-17	F	5	Y (1)
20-Jun-17	F	1	N
20-Jun-17	F	2	N

Table A.3. 2018

Date Collected	**Sex of Bee**	**Number Collected Per Day**	**Stylopized?**
02-May-18	F	1	Y (2)
03-May-18	M	1	Y (1)
03-May-18	M	2	Y (1)
03-May-18	F	3	Y (1)
04-May-18	F	1	Y (2)
04-May-18	M	2	Y (1)
04-May-18	M	3	N
04-May-18	M	4	Y (1)
04-May-18	M	5	N
07-May-18	F	1	N
07-May-18	F	2	N
07-May-18	F	3	N
14-May-18	M	1	N
14-May-18	M	2	Y (Two Males)
15-May-18	M	1	N
15-May-18	F	2	N
15-May-18	M	3	N
16-May-18	M	1	N
16-May-18	F	2	N
16-May-18	F	3	N
16-May-18	F	4	N
16-May-18	F	5	N
18-May-18	F	1	N
18-May-18	M	2	N
18-May-18	M	3	N
18-May-18	M	4	N
18-May-18	F	5	N
18-May-18	F	6	N
18-May-18	M	7	N

22-May-18	F	1	Y (2)
22-May-18	F	2	Y (1)
22-May-18	F	3	N
22-May-18	F	4	N
22-May-18	F	5	N
22-May-18	F	6	N
22-May-18	F	7	N
22-May-18	F	8	N
22-May-18	F	9	N
22-May-18	F	10	N
22-May-18	F	11	N
23-May-18	F	1	N
23-May-18	F	2	N
23-May-18	F	3	N
23-May-18	F	4	N
23-May-18	F	5	N
23-May-18	F	6	N
24-May-18	F	1	N
24-May-18	F	2	N
24-May-18	F	3	N
24-May-18	F	4	N
24-May-18	F	5	N
24-May-18	F	6	N
24-May-18	F	7	N
24-May-18	F	8	N
24-May-18	F	9	N
24-May-18	F	10	N
24-May-18	F	11	N
24-May-18	F	12	N
25-May-18	F	1	Y (1)
25-May-18	F	2	N
25-May-18	F	3	N
25-May-18	F	4	N
25-May-18	F	5	N
28-May-18	F	1	N
28-May-18	F	2	Y (2)
28-May-18	F	3	Y (1)
28-May-18	F	4	Y (1)
28-May-18	F	5	Y (1)
28-May-18	F	6	Y (1)
28-May-18	F	7	N
28-May-18	F	8	Y (1)
28-May-18	F	9	Y (1)
28-May-18	F	10	N
28-May-18	F	11	N

29-May-18	F	1	N
29-May-18	F	2	N
29-May-18	F	3	N
29-May-18	F	4	N
29-May-18	F	5	N
29-May-18	F	6	N
30-May-18	F	1	N
30-May-18	F	2	N
30-May-18	F	3	N
30-May-18	F	4	N
30-May-18	F	5	N
30-May-18	F	6	N
30-May-18	F	7	N
04-Jun-18	F	1	N
04-Jun-18	F	2	N
04-Jun-18	F	3	N
04-Jun-18	F	4	Y (1)
04-Jun-18	F	5	Y (1)
04-Jun-18	F	6	Y (1)
04-Jun-18	F	7	Y (2)
04-Jun-18	F	8	Y (1)
04-Jun-18	F	9	N
04-Jun-18	F	10	N
04-Jun-18	F	11	N
04-Jun-18	F	12	N
05-Jun-18	F	1	N
05-Jun-18	F	2	N
05-Jun-18	F	3	N
05-Jun-18	F	4	N
05-Jun-18	F	5	N
05-Jun-18	F	6	N
05-Jun-18	F	7	Y (1)
05-Jun-18	F	8	N
05-Jun-18	F	9	N
05-Jun-18	F	10	N
05-Jun-18	F	11	N
05-Jun-18	F	12	N
06-Jun-18	F	1	N
06-Jun-18	F	2	N
06-Jun-18	F	3	Y (2)
06-Jun-18	F	4	N
06-Jun-18	F	5	Y (1)
06-Jun-18	F	6	N
06-Jun-18	F	7	N
06-Jun-18	F	8	N

06-Jun-18	F	9	Y (1)
06-Jun-18	F	10	N
06-Jun-18	F	11	Y (1)
06-Jun-18	F	12	N
07-Jun-18	F	1	N
07-Jun-18	F	2	N
07-Jun-18	F	3	N
07-Jun-18	F	4	N
07-Jun-18	F	5	N
07-Jun-18	F	6	N
07-Jun-18	F	7	N
07-Jun-18	F	8	N
08-Jun-18	F	1	N
08-Jun-18	F	2	N
08-Jun-18	F	3	N
08-Jun-18	F	4	N
08-Jun-18	F	5	N
08-Jun-18	F	6	N
08-Jun-18	F	7	N
08-Jun-18	F	8	N
08-Jun-18	F	9	N
08-Jun-18	F	10	N
08-Jun-18	F	11	N
08-Jun-18	F	12	N
08-Jun-18	F	13	N
08-Jun-18	F	14	N
08-Jun-18	F	15	N
11-Jun-18	F	1	Y (1)
11-Jun-18	F	2	Y (1)
11-Jun-18	F	3	N
11-Jun-18	F	4	N
11-Jun-18	F	5	N
11-Jun-18	F	6	N
12-Jun-18	F	1	Y (2)
12-Jun-18	F	2	N
12-Jun-18	F	3	N
12-Jun-18	F	4	N
12-Jun-18	F	5	N
12-Jun-18	F	6	N
12-Jun-18	F	7	Y (1)
12-Jun-18	F	8	N
12-Jun-18	F	9	N
12-Jun-18	F	10	N
12-Jun-18	F	11	N
13-Jun-18	F	1	Y (1)

13-Jun-18	F	2	Y (1)
13-Jun-18	F	3	N
13-Jun-18	F	4	N
13-Jun-18	F	5	Y (1)
13-Jun-18	F	6	Y (1)
13-Jun-18	F	7	Y (1)
13-Jun-18	F	8	Y (3)
13-Jun-18	F	9	N
13-Jun-18	F	10	N
13-Jun-18	F	11	Y (1)
13-Jun-18	F	12	Y (1)
13-Jun-18	F	13	N
14-Jun-18	F	1	N
14-Jun-18	F	2	N
14-Jun-18	F	3	N
14-Jun-18	F	4	Y (1)
14-Jun-18	F	5	N
15-Jun-18	F	1	N
15-Jun-18	F	2	N
15-Jun-18	F	3	N
15-Jun-18	F	4	N
15-Jun-18	F	5	N
15-Jun-18	F	6	N
18-Jun-18	F	1	Y (2)
18-Jun-18	F	2	N
18-Jun-18	F	3	N
18-Jun-18	F	4	N
18-Jun-18	F	5	N
18-Jun-18	F	6	N
19-Jun-18	F	1	N
19-Jun-18	F	2	N
19-Jun-18	F	3	N
19-Jun-18	F	4	N
20-Jun-18	F	1	N
21-Jun-18	F	1	N