Signature page

Microsporidia-host interactions in beneficial lady beetles:

Factors that influence chronic disease.

By
Thomas Steele

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of PhD in Applied Science.

April, 2023, Halifax, Nova Scotia

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Abstract

Lady beetles produce species-specific defensive compounds (alkaloids) that are toxic to some predators and deters others with their foul taste. Some lady beetle alkaloids, such as harmonine from *Harmonia axyridis*, exhibit antimicrobial activity and may provide the beetle protection against pathogens. Convergine and hippodamine are the major and minor alkaloids produced by the convergent lady beetle, *Hippodamia convergens*. Adaline and adalinine are produced by the two-spotted lady beetle, *Adalia bipunctata*. Although the defensive chemistry of these lady beetles has received some attention, the role of their alkaloids with respect to pathogen virulence is poorly understood. Microsporidia, intracellular spore-forming parasites, have been reported in several coccinellid species, including *Tubulinosema hippodamiae* from *H. convergens* and *Vairimorpha* (*Nosema*) *adaliae* from *A. bipunctata*. These pathogens delay larval development but have no other observable effect on the host. However, previous studies were performed under optimal laboratory conditions and did not take into consideration challenges (stress factors) beetles would face in the natural environment. The objective of this study is to determine the relationship between alkaloids and microsporidiosis in *H. convergens* and *A. bipunctata*, and to examine the effects of different stress factors (limited food availability, rearing temperatures above optimal, and physical agitation) on the development of microsporidiosis and production of alkaloids in these beetles. During all three research trials, larval development was delayed for microsporidia-infected beetles when compared to uninfected beetles. In Chapter Two, limited food availability for microsporidia-infected *A. bipunctata* further delayed larval development compared to uninfected beetles, increased pathogen load and reduced adaline content. An irregular, unpredictable food supply acts as a stressor to further amplify the negative effects of microsporidiosis for *A. bipunctata*. No further development delays were observed for *H. convergens* when provided an irregular diet and no conclusions could be made regarding *H. convergens* alkaloid content. In Chapter Three, rearing *A. bipunctata* at temperatures above 25 °C shortened development, decreased pathogen load, and increased adaline content. Temperatures above 25 °C mitigate the microsporidian infection. In the final chapter, adaline content increased as *A. bipunctata* development progressed, uninfected adults produced more adaline than infected beetles when exposed to physical agitation on alternate days, and spore counts were highest when beetles were exposed to daily shaking. As with limited food availability, physical agitation further amplified the negative effects of microsporidiosis in *A. bipunctata* and had an influence on alkaloid production. Many plants produce antimicrobial secondary metabolites (phytoalexins) that can inhibit the germination and/or growth of bacteria and fungi. Alkaloids present in lady beetles may act as the animal equivalent of phytoalexins and play a larger role in the coccinellid immune system than previously thought. These findings provide some preliminary insight into the relationship between adaline, *V. adaliae* and *A. bipunctata*, including certain factors that influence this relationship. However, adalinine, hippodamine and convergine, alkaloids not examined in this study, may also influence infection and could be the focus of future studies.

[27 April 2023]

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Chapter Three

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1. CHAPTER ONE

Introduction

1.1. Lady beetles (Coleoptera: Coccinellidae)

Members of the family Coccinellidae (order Coleoptera), commonly known as ladybirds, ladybugs, or lady beetles, are one of most easily recognizable insect species with their vibrant coloured elytra, typically red, orange, or yellow, and familiar black spots. This family is comprised of more than 6,000 uniquely coloured, dome-shaped species within six subfamilies: Chilocorinae, Coccidulinae, Scymninae, Coccinellinae, Sticholotidinae, and Epilachninae (Majerus, 2009; Omkar, 2016). While not all species display the stereotypical colour pattern, most are brightly coloured with two or more contrasting colors arranged in a bold pattern of spots or stripes (Majerus, 2009).

The typical lady beetle life cycle consists of four stages: egg, larva, pupa, and adult. The life cycle begins when eggs (usually laid as part of a cluster near food sources) hatch to give rise to larvae that develop through four instars before pupation (Dixon, 2000a). Like most insects, the rate of development for lady beetles depends largely on ambient temperature and available food (Dixon, 2000a; Majerus, 2009). However, unlike many other insect species, the larval and adult stages typically feed on the same diet (Majerus, 2009). Lady beetles can be phytophagous, zoophagous or both, as well as generalist or specialist predators (Majerus, 2009; Omkar, 2016). The diet of many predaceous lady beetles includes aphids, coccids, psyllids, diaspids, pentatomids, aleyrodids, and other insects; while non-predaceous species feed on plants, fungi, pollen and honey dew (Omkar, 2016). Most coccinellids are considered natural enemies of other insects (usually considered pests), with exception of the phytophagous subfamily Epilachninae (Omkar, 2016).

Natural enemies either kill or decrease the reproductive potential of pests and are of great ecological importance in pest management because of their host specificity and effectiveness (Flint & Dreistadt, 1998). Many forms of biological control, such as augmentative and classical biological control, exploit this relationship and use mass-produced or field-collected natural enemies for pest control (Wilson & Huffaker, 1976). Augmentative biological control increases the number of natural enemies that already exist in a particular area by releasing large quantities of the same natural enemies or by modifying the local environment to encourage the presence of

natural enemies or increase their effectiveness (DeBach, 1964; Pedigo & Rice, 2006). Classical biological control is effective when the pest species has been introduced, inadvertently or otherwise, into a new ecosystem where their native natural enemies do not exist (Flint et al., 1998; Pedigo & Rice, 2006). Unlike augmentative and classical biological control, Conservation biological control focuses on the protection of existing populations of natural enemies by protecting the local environment within an ecosystem where natural enemies reside (Pedigo & Rice, 2006).

The first observation of lady beetles acting as natural enemies was made by Erasmus Darwin, grandfather to Charles Darwin. Darwin (1800) referred to lady beetles as 'enemies to the aphid' and noted that they 'would appear in their larval state to feed on the pests, change to a chrysalis and then emerge as a small wing-sheathed beetle'. Lady beetles are also responsible for the popularity of modern biological control. In 1878, the developing citrus industry in California was being threatened by a massive infestation of cottony-cushion scale, *Icerya purchasi* Maskell (Riley, 1887). It was determined that the scale had originated from Australia and after foreign exploration for natural enemies of this pest, the vedalia lady beetle, *Rodolia cardinalis* (Mulsant) was imported from New Zealand to the United States (Koebele, 1890; Riley, 1893). Control provided by the vedalia beetle was so successful that cottony-cushion scale was completely controlled in one-and-a-half years and the citrus industry was saved from destruction (Koebele, 1890; Riley, 1893).

Today, several lady beetle species are commercially available for biological control, including the two-spotted lady beetle, *Adalia bipunctata* L. and the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (see Fig. 1.1). These beetles are popular natural enemies because of their ability to consume large amounts of aphids: adult *A. bipunctata* are known to feed continuously, and *H. convergens* can consume their weight in aphids daily as larvae and up to 50 aphids a day as adults (Pedigo & Rice, 2006; Omkar, 2005). *A. bipunctata* is an aphidophagous lady beetle endemic to Europe, Central Asia, and North America (Majerus, 1994). Although this species exhibits a preference for trees and shrubs, *A. bipunctata* can be found in a variety of habitats such as fields, meadows, agricultural crops, grasslands, and woodlands (Leather et al., 1999; Majerus, 1994). Adults exhibit polymorphic colorization that occurs in various forms, ranging from red with two black spots (non-melanic) to black with four or six red spots (melanic) (Omkar, 2005). *H. convergens* is another tree and shrub-dwelling

aphidophagous lady beetle, also found on a variety of vegetation, native to parts of North America (Bouchard, 2014). Convergent lady beetles are named after the two converging white stripes on their pronotum and are characterized by their elongated body with orange elytra and one to six black spots (Bouchard, 2014).

1.2. Lady beetle alkaloids

Lady beetles are easily recognizable due to their brightly coloured elytra and dark spots. This vibrant colouration is an example of aposematic colouration and serves as a warning to potential predators (Hagen, 1962). Most coccinellids reinforce their warning coloration by producing and releasing species-specific alkaloids through reflex-bleeding, a process by which lady beetles secrete a pale-yellow fluid when threatened (King & Meinwald, 1996). This secretion (reflex-fluid) consists of hemolymph and noxious alkaloids (secondary metabolites) that are released from tibiofemoral (leg) joints and pores in the exoskeleton (Majerus, 1994; Weatherson & Percy, 1978). Reflex-fluid may be toxic to other organisms, and it serves as a defense mechanism to protect lady beetles from insect and vertebrate predators (Daloze et al., 1995; Majerus, 1994).

Defining what constitutes an alkaloid is not straightforward and is the subject of some debate. There are different definitions, depending on the discipline, that are considered either too broad or too narrow and often come with a long list of exceptions (Aniszewski, 2015). The term alkaloid was first mentioned by German chemist Carl Friedrich Wilhelm Meißne in 1819, who described these compounds as “like alkali” or “like ashes of plants” (Aniszewski, 2007; Aniszewski, 2015). From a biological perspective, alkaloids were then defined as any heterocyclic chemical compound containing nitrogen, derived from a living organism, that is biologically active and has a pharmacological, medicinal or ecological use (Aniszewski, 2015). However, as more alkaloids were described, researchers recognized that the traditionally accepted definition was not universally accurate, as not all alkaloids contain the stereotypical aromatic ring (see Daloze et al., 1995, King & Meinwald, 1996 and Sloggett et al., 2009 for examples of varying alkaloid forms in coccinellids). Alkaloids are now simply defined as basic nitrogen compounds of naturally occurring organic origins that are mostly heterocyclic (IUPAC, 2019). For organisms that produce alkaloids, these chemicals are recognized as a characteristic feature and can be considered a ‘chemical identification factor’ (Aniszewski, 2007; Hautier et al., 2011).

The first alkaloid to be identified from a coccinellid was the N-oxide coccinelline and its corresponding free base precoccinelline, both extracted from the seven-spotted lady beetle *Coccinella septempunctata* L. (Tursch et al*.,* 1971; Majerus 1994). Since then, more than 50 alkaloids have been characterized from coccinellids of four subfamilies: Coccinellinae (beetles that consume aphids, pollen and fungus), Chilocorinae (beetles that primarily feed on scale), Epilachninae (phytophagous coccinellids), and Scymninae (strictly predatory coccinellids) (Daloze et al., 1995; King & Meinwald, 1996). The chemical structure and biosynthesis of these identified alkaloids have, for the most part, been described (with some debate, see summary in King & Meinwald, 1996).

Subfamily Coccinellinae is the largest and most studied group of lady beetles, from a chemical perspective, and is characterized by the production of alkaloids based on the 2-methylperhydro-9b-azaphenalene ring system (Daloze et al., 1995). The two-spotted lady beetle and convergent lady beetle are both members of Coccinellinae and have been the subject of numerous alkaloid-related studies. Originally, the only alkaloid thought to be present in *A. bipunctata* was the homotropane adaline (Tursch et al., 1973). However, it was later discovered that adaline is accompanied by the minor piperidine alkaloid, adalinine (see Fig. 1.1; Daloze et al*.,* 1995; Lognay et al*.,* 1996). Although adalinine is a piperidone derivative, it is structurally related to the major alkaloid adaline (Lognay et al*.,* 1996). Both adaline and adalinine have been detected in adults, eggs and larval instars of *A. bipunctata*, albeit in unknown quantities (Daloze et al., 1995; Lognay et al*.,* 1996). Four alkaloids have been described from *H. convergens*. Hippodamine and its N-oxide convergine (stereoisomers of coccinelline and precoccinelline, alkaloids from the seven-spotted lady beetle *Coccinella septempunctata*) were identified by Tursch and colleagues in 1972 (Tursch et al., 1972; Tursch et al*.*, 1974). These alkaloids are present in a ratio of roughly 2:1 (convergine: hippodamine) in adult coccinellids and are considered the major and minor alkaloids for *H. convergens* (Tursch et al., 1972). The alkaloids harmonine and n-octylamine were later found in *H. convergens* (see Braconnier et al., 1985; Daloze et al., 1995), presumably at lower levels than hippodamine and convergine. Few coccinellids, not including *H. convergens*, have been studied for their alkaloid content in all life stages throughout development (Daloze et al., 1995). However, because both major and minor alkaloids have been confirmed in *H. convergens* eggs and adults (Kajita et al., 2010), hippodamine and convergine are likely present in all life stages.

When alkaloids were first identified, their synthesis, storage, quantities and diversity within the coccinellid body was unknown. Those studying lady beetle defence chemicals originally hypothesized that lady beetles biosynthesized and stored their defensive chemicals using the polyketide pathway in complex glands at the base of each leg (Dettner, 1987). Later studies determined that alkaloids are distributed throughout all parts of adult lady beetles but occur at higher concentrations in reflex fluid (de Jong et al., 1991; Holloway et al*.,* 1991), and that these secondary metabolites are biosynthesized through a fatty acid pathway in the insect fat body (Laurent et al., 2002). Researchers also hypothesized that coccinellids only produced a single alkaloid or alkaloid-pair, but it has since been demonstrated that the alkaloid content of lady beetles is more complex (Daloze et al., 1995). Lady beetles can possess a single alkaloid or a complex mixture of several different alkaloids; however, one to two primary alkaloids are typically responsible for the majority composition of their reflex-fluid (Pasteels, 2007).

All coccinellid alkaloids exhibit some repellent abilities; however, the efficiency and severity depends on a variety of factors, including age, sex, species, toxicity and concentration (Daloze et al., 1995; Pasteels, 2007). For example, adult *A. bipunctata* males produce significantly less alkaloids than females, younger adults and those that are overwintering (de Jong et al., 1991). *A. bipunctata* also produces higher alkaloid concentrations in hemolymph than does *C. septempunctata*, but the alkaloids found in *C. septempunctata* offer superior protection against predators (Marples, 1993a). In *H. convergens*, alkaloid concentrations vary depending on sex as well as colouration; the reddest females contain the highest concentrations of alkaloids (Wheeler et al., 2015).

Alkaloids from numerous lady beetle species exhibit repellent activity against ants. The repellent characteristic of these chemicals has been reported from the Mexican bean beetle, *Epilachna varivestis* (Attygale et al., 1993); the South American ladybird beetle, *Epilachna paenulata* (Camarano & González, 2006); the whitefly predator, *Delphastus catalinae* (Deyrup et al., 2014), the two-spotted lady beetle, *A. bipunctata* (Hill & Renbaum, 1982), the seven-spotted lady beetle, *C. septempunctata* (Marples, 1993a; Pasteels et al., 1973); the fourteen-spotted lady beetle, *Propylea quatuordecimpunctata* (Marples, 1993b, Pasteels et al., 1973); the twenty-two spot lady beetle, *Psyllobora vigintiduopunctata* (Marples, 1993b; Pasteels et al., 1973); and the multicolored Asian lady beetle, *Harmonia axyridis* (Sloggett et al., 2011). However, the degree to which these beetles are chemically defended varies between species

(Marples, 1993a; Pasteels et al., 1973). Generally, when ants encounter reflex-fluid they respond by immediately backing away from the beetle and they begin to intensively clean their bodies because the alkaloid-containing hemolymph coagulates quickly and becomes sticky, gluing body parts of the ants together. Lady beetles appear to be immune to these effects and can easily remove dried reflex-fluid (Happ & Eisner, 1961).

Alkaloids act as multifunctional defense chemicals, serving as toxicants, insecticides, surfactants and antimicrobial agents (Dettner, 1987). A lot of the attention in this research area has focused the alkaloids of *Harmonia axyridis*. Harmonine, the major alkaloid purified from *H. axyridis*, has shown broad-spectrum antimicrobial activity against several human pathogens, including: a drug-sensitive *Staphylococcus aureus*; a multi-resistant *S. aureus* (MRSA) strain; four strains of fast-growing mycobacteria, including *Mycobacterium tuberculosis* responsible for tuberculosis; and the asexual blood stages of *Plasmodium falciparum*, the pathogen responsible for malaria tropica (see Röhrich et al., 2012). Synthetic harmonine induces necrotic cell death in *Leishmania major*, a pathogen responsible for cutaneous leishmaniasis (Nagel et al., 2015). Reflex-fluid from *H. axyridis* also exhibits antimicrobial activity against *Escherichia coli* (Kogel et al., 2012). Compounds isolated from *H. axyridis* alkaloids demonstrate significant cytotoxicity against five human tumor cell lines as well as some minor inhibitory ability against enzymes linked to Alzheimer's disease (Alam et al*.,* 2002).

These preliminary trials of reflex-fluid and purified alkaloids against other arthropods, as well as antimicrobial studies against human pathogens, have all shown some degree of repellent and antimicrobial potential. However, the role of alkaloids with respect to pathogen virulence, specifically that of microsporidia in lady beetles, is poorly understood.

1.3. Microsporidia

A growing area of concern with regards to the overall success of biological control is the quality and condition of the natural enemies used (van Lenteren, 2003). It is well known that lady beetles host a wide variety of symbionts, ranging from invertebrate parasites to microorganisms. The list of parasitoids and pathogens that affect lady beetles includes flies (*Phalocrotophora* spp., *Medina* spp.), wasps, (*Dinocampus coccinellae*, *Homalotylus* spp., Tetrastichinae spp., Pediobius *foveolatus*), mites and ticks (*Coccipolipus* spp.), nematodes (*Parasitilenchus coccinellinae*, *Howardula* spp., Mermitidae spp.), protozoans (gregarines), fungi

(Hyphomycetes, microsporidia), male-killing bacteria (*Wolbachia, Rickettsia* and *Spiroplasma*) and several viruses (see Ceryngier & Hodek, 1996; Riddick et al., 2009, Richerson, 1970).

Microsporidia are small, obligate intracellular eukaryotic pathogens that produce unicellular spores for transmission (Bulla & Cheng, 1976; Tanada & Kaya, 1993). Microsporidia are highly specialized to depend on their hosts and are characterized by a dramatic reduction of cellular components, such as the mitochondria (Keeling & Fast, 2002; Texier et al., 2010). This evolved dependence between microsporidia and their hosts has resulted in modifications to pathogen ultrastructure that are suited for intercellular pathogen transmission, and changes in biochemistry, metabolism and a reduced genome (Keeling & Fast, 2002).

The microsporidian lifecycle has two distinct phases: (1) merogony, the vegetative stage that involves the production and multiplication of developmental stages and (2) sporogony, the production of transmissible spores (Tanada & Kaya, 1993). Microsporidia inoculate new tissues using a unique penetration structure, called a polar filament. Environmental stimuli, such as a change in pH or osmotic pressure, causes the posterior vacuole to swell and results in an increase in internal pressure inside the microsporidian spore, which then causes the polar filament to discharge rapidly (Tanada & Kaya, 1993). During this process, the polar filament everts to form a hollow tube that works like a needle to inject the infective material into the new host cell (Keeling & Fast, 2002; Tanada & Kaya, 1993; Xu & Weiss, 2005).

The simplistic cellular structure of microsporidia has made it difficult to determine the evolutionary relationship of these pathogens to other eukaryotes (Corradi & Keeling, 2009). Microsporidia were originally described as yeast-like fungi; however, discovery of the unique mode of infection then led researchers to classify them in Phylum Microsporidia, separate from the fungi (Keeling & Fast, 2002). The taxonomic classification of microsporidia has since changed numerous times, but results from molecular analyses have suggests a relationship between microsporidia and fungi; however, the exact relationship has yet to be determined (Corradi & Keeling, 2009; Keeling & Fast, 2002; Keeling, 2009).

Microsporidia infect a large range of hosts, including all five classes of vertebrates, most invertebrate phyla and some protists (Bulla & Cheng, 1976). More than 1300 species of microsporidia have been described within 160 genera (Lacey & Kaya, 2007). The most common hosts are arthropods, with insects being type hosts of 90 microsporidian genera (Solter et al., 2012). Microsporidia were once thought to be host specific and were identified based on their

host. This has caused some taxonomic confusion because some microsporidia are now known to infect more than one host (Tanada & Kaya, 1993), including microsporidia from coccinellids (Saito & Bjørnson, 2008; Steele & Bjørnson, 2012).The description of new species now includes ultrastructure characteristics and molecular phylogeny (Vossbrinck & Debrunner-Vossbrinck, 2005; Vossbrinck et al., 2014); however, the reduced genome and organelles in microsporidia has contributed to some taxonomic confusion. For example, the genera *Nosema* and *Vairimorpha* both contain species that were originally classified using a limited understanding of molecular and cellular characteristics. Some species originally classified in the *Nosema* genus lacked characteristic developmental stages during sporogony that had been traditionally assigned to the genus *Vairimorpha*, and vice versa (other species assigned to *Vairimorpha* possessed these characteristic stages). Molecular analyses has since revealed that microsporidia from both genera can lack or exhibit these characteristic stages during sporogony. This adds to the confusion when the genome of a new species is compared to a previously described species that had been initially assigned to the incorrect genus (see Tokarev et al., 2020 for a complete description of the redefinition and reassignment of these genera).

Microsporidia are transmitted through three natural entry points: oral, cuticular and ovarial (Kramer, 1976). Transmission by the oral and cuticular pathways results in horizontal transmission whereas vertical transmission is achieved through the ovarial portal (Agnew, 2003). Symptoms of microsporidian infections vary greatly depending on pathogen and host; however, most infections tend to be sublethal and chronic (Solter et al., 2012). Insects infected with microsporidia may be altered in colour, size, form and activity, have delayed growth and reduced size prior to death (Tanada & Kaya, 1993). Other symptoms include abnormal feeding, incomplete metamorphosis, deformed pupae and adults, and lowered fecundity (see Bjørnson & Oi, 2014). In economically important insects, both beneficial and pest species, microsporidia can cause economically serious diseases (microsporidiosis) (Keeling, 2009; Tanada & Kaya, 1993).

To date, six species of microsporidia have been described from lady beetles, including *Vairimorpha* (*Nosema*) *adaliae* from the two-spotted lady beetle, *A. bipunctata* (Steele & Bjørnson, 2014) and *Tubulinosema hippodamiae* from the convergent lady beetle, *H. convergens* (see Fig. 1.1; Bjørnson et al., 2011). In their natural hosts, both pathogens significantly delay larval development, but have no effect on mortality, fecundity, longevity, or sex ratios (Saito & Bjørnson, 2008; Steele & Bjørnson, 2012). However, these microsporidia can infect other

coccinellids under laboratory conditions with unpredictable and inconsistent effects. For example, *V. adaliae* from *A. bipunctata* can infect *H.* convergens, but the pathogen has no effect on larval development or mortality (Steele & Bjørnson, 2012). In contrast, when *T. hippodamiae*, from *H. convergens,* infects the seven-spotted lady beetle, *Coccinella septempunctata,* and the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, larval development is prolonged (Saito & Bjørnson, 2008). These inconsistencies raise questions regarding the factors that can influence a particular pathogen to produce chronic, unpredictable effects in related, susceptible hosts.

1.4. Stress factors

Because of their short life cycle and rapid reproduction, insects are easily influenced by changes in their natural environment from abiotic (temperature, humidity, light) and biotic (food availability, crowding) stress factors (Khaliq et al., 2014). It is difficult to generalize the responses of insects and insect populations to environmental stressors as these responses are determined by the type of stress, the particular insect species affected, and the stage at which the insect is exposed (Pimentel, 1993).

The effects of environmental stressors on lady beetles are well documented. When provided with reduced, artificial or suboptimal prey, *A. bipunctata* and *H. convergens* adults take longer to develop, produce fewer eggs, weigh less and have higher mortality rates (Bonte et al., 2010; Cottrell et al, 2017; Hussein & Hagen, 1991; Jalali et al., 2009). Development rates for both *A. bipunctata* and *H. convergens* are positively correlated with an increase in temperature that is negatively correlated with mortality rates (Jalali et al., 2009; Rodriguez-Saona & Miller, 1999). However, these coccinellid species fail to develop fully to adults when temperatures exceed 30 °C (Jalali et al., 2009; Rodriguez-Saona & Miller, 1999). For many coccinellids, overcrowding functions as an indicator to switch their focus from reproduction to dispersal, to mitigate stress (Dixon & Agarwala, 1999). Overcrowded lady beetles often resort to competition and cannibalism as food resources become limited (Dimetry, 1976; Dixon & Agarwala, 1999).

Despite efforts to provide and maintain optimal conditions in insect mass-rearing, stress factors such as suboptimal nutrition, temperature, and crowding are often unavoidable and can increase the susceptibility of many insects to disease (James et al., 1998; James & Lighthart, 1992; Steinhaus, 1958). For example, green lacewings (*Chrysoperla carnea*) and red flour

beetles (*Tribolium castaneum*) are more susceptible to the fungal pathogen *Beauveria bassiana* when exposed to stress factors such as temperature, starvation and suboptimal nutrition (Donegan & Lighthard, 1989; Lord, 2010). Several caterpillar species, including the alfalfa caterpillar, *Colias* (*philodice*) *eurytheme* and the buck-eye caterpillar, *Junonia coenia* show increased mortality rates and susceptibility to various naturally occurring pathogens when reared under overcrowded conditions (Steinhaus, 1958).

Although insufficient food availability, temperature extremes and crowding are recognized as a stress factor that reduce the fitness of many insects, including coccinellids, the consequences of these stress factors when *A. bipunctata* and *H. convergens* are infected with a microsporidian pathogen have not been investigated.

1.5. Objectives

Because microsporidia lack mitochondria and are completely dependent on their host for energy, one would expect these pathogens to produce infections in coccinellids that are more damaging to the host than what has been documented. Previous studies of microsporidiosis in lady beetles have focused on the effects of microsporidia on beetles that are maintained in optimal laboratory conditions but did not take into consideration the impacts of biotic or abiotic challenges that beetles often face in the natural environment. Some of these challenges, such as overcrowding, physical agitation, temperature variations and inadequate food have been shown to increase the susceptibility of insects to other fungal and bacterial infections. Additionally, the impact of these biotic and abiotic pressures (stress factors) on the production of coccinellid alkaloids remains vastly unexplored. Lady beetle alkaloids are known to protect beetles from predators; however, some coccinellids alkaloids, such as harmonine, exhibit antimicrobial activity against specific protozoan (*P. falciparum, L. major*), and bacterial (*M. tuberculosis, E. coli*) pathogens. With this is in mind, lady beetle alkaloids may also play a role in protecting coccinellids from certain insect pathogens.

The aim of this study is to investigate the influence of alkaloids in the two-spotted lady beetle, *Adalia bipunctata* (adaline and adalinine), and the convergent lady beetle, *Hippodamia convergens* (convergine and hippodamine), on two microsporidian pathogens that cause chronic microsporidiosis in these hosts (*Vairimorpha adaliae* and *Tubulinosema hippodamiae*,

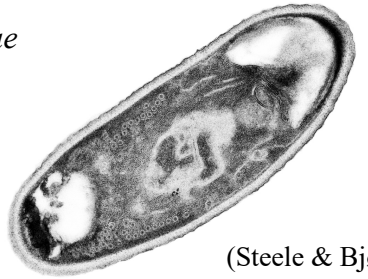
respectively). The relationship between alkaloid production, chronic microsporidiosis, and certain stress factors will be investigated as follows:

1. The effect of food availability on alkaloid production and microsporidiosis in *A. bipunctata* and *H. convergens*. The impact on larval development and mortality will be examined, and adult morphometrics and pathogen spore load will be quantified.
2. The effect of temperature on alkaloid production and microsporidiosis in *A. bipunctata*. The impact on larval development and mortality will be examined, and adult morphometrics and pathogen spore load will be quantified.
3. The effects of microsporidiosis on alkaloid content in *Adalia bipunctata* development and the influence of physical stress on alkaloid production and microsporidiosis in adult *A. bipunctata*. All life stages will be examined for alkaloid content and pathogen spore loads will be quantified in adults following exposure to varying amount of physical agitation.

(a) *Adalia bipunctata*

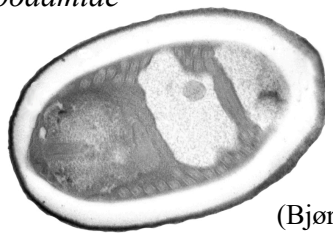
(b) *Hippodamia convergens*

Vairimorpha adaliae



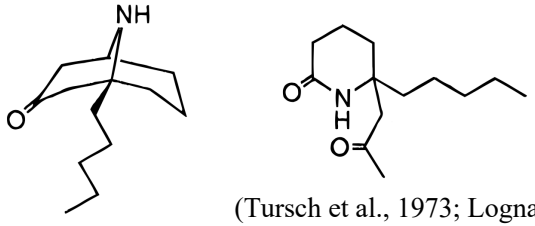
(Steele & Bjørnson, 2014)

Tubulinosema hippodamiae



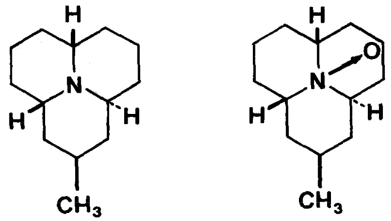
(Bjørnson et al., 2011)

Adaline (L) & Adalinine (R)



(Tursch et al., 1973; Lognay et al., 1996)

Hippodamine (L) & Convergine (R)



(Tursch et al., 1974)

Figure 1.1. Coccinellid species, microsporidia and alkaloids that are the subject of this study. The two-spotted lady beetle, *Adalia bipunctata* (**a**), is host to the microsporidium *Vairimorpha adaliae* and produces the major and minor defensive alkaloids adaline and adalinine, respectively. The convergent lady beetle, *Hippodamia convergens* (**b**), is host to the microsporidium *Tubulinosema hippodamiae* and produces the major and minor alkaloids convergine and hippodamine, among others.

2. CHAPTER TWO

Effects of microsporidiosis and food availability on the two-spotted lady beetle, *Adalia bipunctata* L., and convergent lady beetle, *Hippodamia convergens* Guérin-Méneville

2.1. Introduction

The two-spotted lady beetle, *Adalia bipunctata* L., and convergent lady beetle, *Hippodamia convergens* Guérin-Méneville, are used in biological pest control programs because they have a voracious appetite for aphids (Okmar, 2005; Pedigo & Rice, 2006). Like most commercially-available natural enemies, *A. bipunctata* is mass-reared. The consistent production of large numbers of natural enemies in a commercial insectary requires optimal rearing conditions (Boller & Chambers, 1977). Careful consideration is given to optimize nutrition (food sources and quantities, artificial diets) and environment (temperatures, light cycles and substrates). To prevent disease outbreaks, quarantine procedures are recommended to ensure that pathogens are excluded from mass-rearings (van Lenteren, 2003). Unlike *A. bipunctata*, *H. convergens* is not mass-produced, but are collected annually from their overwintering sites in California. As a result, there is no means or incentive to implement or maintain quality control measures for this natural enemy.

Both *A. bipunctata* and *H. convergens*, like many lady beetle species, display aposematic (warning) colouration that is reinforced by the production of species-specific alkaloids (see review by King & Meinwald, 1996). Alkaloids are unpalatable, sometimes toxic, defense chemicals used to deter predators (Daloze et al., 1995; Majerus, 1994). Because alkaloids also exhibit antimicrobial properties, they act as multi-functional defense chemicals that are suspected to play a role in lady beetle immune processes (Dettner, 1987; Kogel et al., 2012; Röhrich et al., 2012). Two alkaloids have been described from *A. bipunctata*: the major homotropane alkaloid adaline (Tursch et al., 1973), and the minor piperidine alkaloid adalinine (Lognay et al., 1996). Two azaphenalene alkaloids have been isolated from *H. convergens*: the major alkaloid convergine and the minor alkaloid hippodamine (Tursch et al., 1972; 1974). The role of these alkaloids, with respect to the development of disease in lady beetles, has yet to be established.

Lady beetles host a wide variety of pathogens, parasites and parasitoids, including microsporidia (Riddick et al., 2009). Six species of microsporidia have been described from lady beetles, including *Vairimorpha* (*Nosema*) *adaliae* from *A. bipunctata* (Steele & Bjørnson, 2014)

and *Tubulinosema hippodami*ae from *H. convergens* (Bjørnson et al., 2011). In the case of *A. bipunctata*, larval development is prolonged significantly by *V. adaliae*, but the pathogen has no effect on mortality, fecundity, longevity, or sex ratios (Steele & Bjørnson, 2012). *T. hippodamiae* has similar effects on *H. convergens* (Saito & Bjørnson, 2008).

Some microsporidia from lady beetles can infect other coccinellids under laboratory conditions with unpredictable and inconsistent outcomes. For example, *V. adaliae* (natural host: *A. bipunctata*) infects *H. convergens* but has no effect on larval development or mortality (Steele & Bjørnson, 2012). However, when *T. hippodami*ae (natural host: *H. convergens*) infects the seven-spotted lady beetle, *Coccinella septempunctata* L., and the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, larval development is prolonged but larval mortality is unaffected (Saito & Bjørnson, 2008). Another microsporidium, which is closely related to *Nosema thompsoni*, produces abundant spores in *H. axyridis* without causing mortality (Vilcinskas et al., 2013). However, when *C. septempunctata* is infected with the same pathogen, all individuals die within two weeks. Such inconsistencies raise questions regarding the factors that influence a particular pathogen to produce unpredictable effects in related, susceptible hosts.

Despite efforts to provide and maintain optimal conditions in insect mass-rearings, stress factors such as temperature, crowding and suboptimal nutrition, are often unavoidable and increase the susceptibility of many insects to disease (James et al., 1998; James & Lighthart, 1992; Steinhaus, 1958). For example, green lacewings (*Chrysoperla carnea* Stephens) and red flour beetles (*Tribolium castaneum* Herbst) are susceptible to the fungal pathogen *Beauveria bassiana* when nutrition is suboptimal (Donegan & Lighthart, 1989; Lord, 2010). When provided with reduced, artificial or suboptimal prey, *A. bipunctata* and *H. convergens* take longer to develop, produce fewer eggs, weigh less and have higher mortality rates (Bonte et al., 2010; Cottrell & Tillman, 2017; Hussein & Hagen, 1991; Jalali et al., 2009). Although insufficient food availability is recognized as a stress factor that reduces fitness in *A. bipunctata* and *H. convergens*, the consequences of an irregular food supply when beetles are infected with a microsporidian pathogen have not been investigated. The objective of this study was to examine the effects of reduced food availability and microsporidiosis on larval development, alkaloid content (relative percent alkaloid in reflex-fluid, RP adaline), and adult morphometrics (body measurements) of *Adalia bipunctata* and *Hippodamia convergens* when infected with *Vairimorpha adaliae* and *Tubulinosema hippodamiae*, respectively.

2.2. Materials and methods

Uninfected *A. bipunctata* and *H. convergens* used in this study were selected from established laboratory colonies. *Vairimorpha adaliae* and *Tubulinosema hippodamiae* were sustained in isolated colonies of *A. bipunctata* and *H. convergens*, respectively.

Uninfected and microsporidia-infected stock beetles were reared in clear 120-mL polyethylene cups (Canemco-Marivac Inc., QC). Each cup had a 2.5-cm hole cut in the side that was covered with fine mesh for ventilation. Stock beetles were maintained on artificial diet (Lacewing and Ladybug food, Planet Natural; prepared with equal parts honey). Water was provided daily through a moistened cotton wick (Crosstex International, NY).

To obtain uninfected eggs and test larvae for the study, eight mating pairs of each beetle species were isolated from uninfected stock beetles and reared in polyethylene cups. To acquire microsporidia-infected eggs, another eight mating pairs were isolated from infected *A. bipunctata* and *H. convergens* stock beetles. Parent beetles were fed a diet of green peach aphids (*Myzus persicae* Sulzer) that were reared on nasturtium (*Tropaeolum minus*, Dwarf Jewel Mixed; Stokes Seed Ltd., ON), supplemented with artificial diet. Eggs were collected daily.

The infection status of all test larvae, and the eggs that were provided to them, was confirmed by examining a subsample of sibling larvae and eggs from each mating pair by light microscopy. Eggs and larvae were randomly selected from each mating pair, smeared on a microscope slide, stained with 5% Giemsa (Sigma-Aldrich, MO) and examined for the presence of microsporidian spores (40× magnification). It is difficult to assess spore counts in eggs before they are fed to test larvae; however, a single microsporidia-infected egg is enough to transmit *V. adaliae* or *T. hippodamiae* to susceptible coccinellid hosts (Saito & Bjørnson, 2008; Steele & Bjørnson, 2012). At the end of the trial, each parent beetle was examined for spores to confirm infection status post-mortem. Plants, aphids, larvae and beetles were maintained in separate environmental chambers (Sanyo MLR-350H) under controlled conditions (16:8 L:D; 25 °C:20 °C).

2.2.1. Larval development

To determine the effects of irregular food availability (limited prey) on the development of microsporidia-infected lady beetles, test larvae were allocated to four groups based on infection status and food availability: uninfected/daily, microsporidia-infected/daily, uninfected/irregular,

and microsporidia-infected/irregular (Table 2.1). Uninfected *A. bipunctata* and *H. convergens* larvae (24-h old, unfed) were individually isolated in Petri dishes (47-mm diameter, Millipore Corp., MA). A 3-cm hole in the lid of each dish had been covered in mesh to provide air circulation. For the uninfected groups (both daily and irregular diets), three test larvae were isolated daily for 4 days (n = 12). For the microsporidia-infected groups (both daily and irregular diets), six individuals were isolated for 6 days (n = 36). Fewer uninfected larvae were isolated because larval development data for *A. bipunctata* and *H. convergens* is well reported in the literature under both optimal and suboptimal feeding conditions. Water was provided through a cotton wick and one conspecific egg was provided to each test larva (either uninfected or microsporidia-infected, depending on species and treatment; Table 2.1) on a filter paper disk (6-mm diameter) that was placed in the center of the dish. Larvae that consumed the egg within 24-h were then provided with either an ample supply of aphids daily (uninfected/daily, infected/daily) or an ample supply of aphids on specific days only (uninfected/irregular, infected/irregular). The latter diet consisted of one day of aphids supplied in abundance, followed by one or two consecutive days when food was not provided. On days when food was not provided, a fine artist's brush was used to remove any aphids that had been placed in the dish on the previous day but had not been eaten. The irregular feeding schedule for these larvae was designed to represent suboptimal and inconsistent prey availability that can be experienced in nature, and to simulate periods of temporary food deprivation individuals may encounter due to, weather extremes, competition, habitat loss and changes in environmental conditions. The two feeding schedules were implemented until all test larvae emerged as adults at the end of the trial.

Larvae that consumed the conspecific egg within 24-h were provided aphids on Day 1 of the trial. Dietary restrictions were implemented on Day 2, according to the schedule outlined above. Larvae were observed daily, and development and mortality were recorded. After the larvae completed development and emerged as adults, they were given water and allowed to rest undisturbed for 24-h before being sexed. This trial was repeated with new mating parents isolated from stock colonies. Mean development time (days) was calculated for individuals that completed larval development and emerged as adults. Data was excluded for larvae that died prematurely or did not eat the conspecific egg that was provided within the initial 48-h. A two-way ANOVA (SPSS Statistics, IMB) was used to compare the duration of larval development

between treatments, and to identify any interactions between infection status and/or food availability on larval development.

2.2.2. Larval mortality and adult sex ratio

Larvae, pupae, and adults that died during the trial were smeared, stained with Giemsa and examined by light microscopy for the absence or presence of microsporidian spores. A chi-square test (SPSS Statistics, IMB) was used to analyze differences in larval mortality between groups. Adult beetles were sexed 24-h after eclosion by examining their terminal, abdominal sternites under a stereomicroscope. Beetles were handled gently with soft-forceps and care was taken to prevent and/or minimize reflex-bleeding. A chi-square test was used to analyze sex ratio differences. Adult beetles were given artificial diet and water and allowed to rest undisturbed for an additional 24-h before a sub-sample was selected at random for body measurements. This additional 24-h rest period was included to ensure that the body was fully sclerotized before measurements were taken. When adults were 48-h old, each was smeared, stained with Giemsa, and examined by light microscopy to confirm infection status.

2.2.3. Alkaloids

At the end of the trial, adult beetles were given artificial diet and water and allowed to rest undisturbed for an additional 24-h after sexing before a sub-sample of 10 beetles (five female and five male) from each group was selected at random to collect alkaloid samples and take body measurements. The 24-h period was included to ensure that the body was fully sclerotized before the beetles were handled and that the beetles were undisturbed before collecting reflex-fluid. Immediately prior to being photographed for microscopic measurements, alkaloid samples were taken. Beetles were held by the abdomen with soft forceps, then softly prodded with a metal probe until a drop of reflex-fluid was released from the tibiofemoral joint. Reflex-fluid was collected with a sterile 2-µl glass capillary micropipette, and immediately following collection, each micropipette was submerged in a separate vial that contained 0.5 ml of methanol. Vials were stored in a freezer (−20 °C) for 6 months until analysis. To confirm the stability of these alkaloids during storage, fresh reflex-fluid samples were collected and analyzed alongside those collected earlier and results from the fresh samples were compared to those that had been stored.

Unfortunately, samples collected from *H. convergens* had degraded over time and were unable to be analyzed for alkaloid comparison (convergine and hippodamine).

Gas chromatography (GC) was used with mass spectrometry (MS) for alkaloid identification and flame ionization detection (FID) to determine alkaloid concentrations in *A. bipunctata* (proportion of adaline relative to other components of reflex-fluid). Samples were analyzed on a Varian 3800 series gas chromatograph (Agilent Technologies, Santa Clara, CA), equipped with a VF-5ms capillary column (30 m × 0.25 mm; CP8944; Agilent J&W GC Columns, Santa Clara, CA) and FID. The GC injection port was set to 240 °C and samples were injected in both split (MS) and splitless (FID) modes with a split ratio of 0.5. Half a microliter of sample (reflex-fluid in MeOH) was injected into the GC using a Varian CP-8400 autosampler. Column temperature was set to 50 °C with an increase of 20 °C per minute and a hold of 4-min. The maximum temperature was 330 °C with a hold of 5-min. The FID detector was set to 330 °C. Helium was used as a carrier gas at a flow of 1 ml/min. The GC was interfaced with a Varian Saturn 2000 series GC/MS/MS with electron impact ionization (ionization energy of 70 eV; 20–400 m/z scan range). Adaline was identified by matching mass spectra with those in the literature (Hautier et al., 2008). Saturn GC/MS Workstation (version 6.30) software was used to determine the area of all peaks in each sample. The ratio of each adaline peak (presented as relative % area of adaline peak), relative to other components of reflex-fluid (total area of all peaks), was calculated. Methanol peaks were excluded from the calculations so that proportion of adaline was compared to reflex-fluid only. The response of adaline in each sample was expected to be the same. Averages for each treatment were calculated and a two-way ANOVA with a Tukey's post-hoc test (SPSS Statistics, IMB) was used to compare mean relative proportions of adaline between groups.

2.2.4. Adult body measurements

Before beetles were smeared to confirm infection status, 10 adults (five female and five male) from each group were photographed with a Zeiss Axio Imager A1 light microscope and attached Zeiss AxioCam MRc digital camera. Body measurements (elytra length, pronotum width, femur length and head capsule width) were taken using the AxioVision software (v 4.6.3) measurement tool. Length is correlated to body weight for most insect species (Rogers et al., 1976; Hódar, 1997); therefore, body measurements were used *in lieu* of weight to determine fitness. Multiple

body measurements were used for size analyses because they provide more reliable information than single measurements (Knapp & Knappová, 2013). Because sclerotized body parts are the most reliable indicators of size (Daly, 1985; Knapp & Knappová, 2013), the width of the pronotum and head capsule, and the length of the elytra and hind-femur were measured. Six photographs were taken of each adult (three of the anterior regions and three posterior) from which measurements were taken and means calculated for each body part. A two-way ANOVA (SPSS Statistics, IMB) was used to compare body measurements between groups, and to identify any interactions between body measurements, and infection status or food availability.

2.2.5. Pathogen load

After collection of reflex-fluid, the same sub-samples of beetles were smeared on glass microscope slides and stained with Giemsa (5%), and these were examined for microsporidian spores using light microscopy. Five areas of each microscope slide were selected at random for examination with a Zeiss Axio Imager A1 light micro- scope equipped with an ocular grid reticle at 100x objective (oil-immersion, 1000x magnification). Spores in each grid were counted, and mean spore load per area (120 μm^2) was calculated for each beetle. A two-sample t-test with equal variances (SPSS Statistics, IMB) was used to compare mean spore counts.

2.3. Results

Microsporidian spores were not detected in smear preparations made from parent beetles that originated from uninfected beetle stock. These had been used to produce test larvae and uninfected eggs that were fed to test larvae. Conversely, microsporidian spores were detected in all parent beetles that produced infected eggs that were fed to test larvae.

At the end of the trial, spores were not detected in male or female beetles, or in larvae that failed to complete development from the control groups. However, microsporidian spores were detected in all individuals that had been fed microsporidia-infected eggs as 24-h old larvae, with the exception of two larvae (one *A. bipunctata* and one *H. convergens*) that died within the initial 48-h of the study and these were excluded from the data analyses.

2.3.1. Larval development

Mean development for *A. bipunctata* that consumed an uninfected *A. bipunctata* egg was 17.00 and 23.62 days, for those fed daily and irregularly, respectively (Table 2.2). Mean larval development was 17.92 and 26.06 days for larvae fed *V. adaliae*-infected eggs (fed daily and irregularly, respectively). Development time of *A. bipunctata* larvae that consumed *V. adaliae*-infected eggs was significantly longer than those that ate uninfected eggs ($F = 20.86$; $df = 1$; $p < 0.001$). Development was also delayed for larvae that were provided an irregular diet of aphids, compared to those fed daily ($F = 402.89$; $df = 1$; $p < 0.001$). There was a significant interaction between infection status (uninfected vs. microsporidia-infected) and food availability (fed daily vs. fed irregularly) ($F = 4.30$; $df = 1$; $p = 0.040$; Table 2.2).

Mean developmental for *H. convergens* larvae that ate an uninfected *H. convergens* egg was 17.57 and 27.75 days, for those fed daily and irregularly, respectively (Table 2.3). Mean larval development was 18.38 and 29.51 days for larvae fed *T. hippodamiae*-infected eggs (fed daily and irregularly, respectively). Larval development for *H. convergens* fed *T. hippodamiae*-infected eggs was significantly longer than that of larvae fed uninfected eggs ($F = 10.85$; $df = 1$; $p < 0.001$). Development was also delayed for larvae that were fed irregularly when compared to larvae fed daily ($F = 743.40$; $df = 1$; $p < 0.001$). However, there was no significant interaction between infection status (uninfected vs. microsporidia-infected) and food availability (fed daily vs. fed irregularly) on larval development ($F = 1.47$; $df = 1$; $p = 0.228$; Table 2.3).

2.3.2. Larval mortality and adult sex ratio

For *A. bipunctata*, larval mortality ranged from 0% to 47.06%. Although these values differ considerably, they did not differ significantly ($\chi 2 = 1.890$, $df = 1$, $p = 0.169$; Table 2.2). A similar trend was observed for *H. convergens*. Although *H. convergens* larval mortality ranged from 4.17% to 55.56%, these results did not differ significantly ($\chi 2 = 0.608$, $df = 1$, $p = 0.435$; Table 2.3). For both beetle species, sex ratios were close to 1:1 (♀:♂) and did not differ significantly between groups for *A. bipunctata* ($\chi 2 = 6.89$, $df = 3$, $p = 0.075$) or *H. convergens* ($\chi 2 = 0.371$, $df = 3$, $p = 0.946$).

2.3.3. Alkaloids

Adaline content differed significantly between uninfected and microsporidia-infected individuals ($F = 23.41$, $df = 1$, $p < 0.001$) and between those that were fed daily compared to those fed irregularly ($F = 10.10$, $df = 1$, $p < 0.001$). A significant interaction between infection status and food availability was observed with respect to the relative percent of adaline in reflex-fluid ($F = 4.53$, $df = 1$, $p = 0.04$; Fig. 2.1). When uninfected beetles were provided a daily diet of green peach aphids, the mean relative percent of adaline was 27.23 ± 2.21, whereas infected beetles fed daily had significantly higher levels of adaline in their reflex fluid (35.53 ± 3.17 relative percent). When food was supplied on an irregular basis, a similar trend was observed; mean relative percent adaline was 13.68 ± 3.57 in uninfected beetles and 32.51 ± 2.16 in microsporidia-infected beetles. However, compared with beetles fed a daily diet, the difference in adaline content of the reflex fluid was much greater between irregularly fed uninfected and *V. adaliae*-infected *A. bipunctata*. The minor alkaloid adalinine was not detectable with the current collection method and GC–MS analysis. Summary data for all adaline peaks, and examples of the typical output data provided by the software, are available in the Appendix.

2.3.4. Adult body measurements

For *A. bipunctata*, body measurements of male and female beetles did not differ significantly, nor did the interaction between sex and infection status ($p > 0.05$; Table 2.4). A significant interaction was observed between food availability and sex on hind femur length ($F = 5.55$; $df = 1$; $p = 0.025$). For individuals that were provided a daily supply of aphids, all body measurements were significantly greater than those from individuals that were fed irregularly (elytra: $F = 32.73$, $df = 1$, $p < 0.001$; pronotum: $F = 31.001$, $df = 1$, $p < 0.001$; femur: $F = 35.21$, $df = 1$, $p < 0.001$; head capsule: $F = 16.98$, $df = 1$, $p < 0.001$). Mean elytra length was significantly less for *A. bipunctata* larvae that consumed an uninfected egg when compared to larvae that consumed an *V. adaliae*-infected egg ($F = 5.08$, $df = 1$, $p = 0.032$), but differences were not observed for the other body parts measured. No significant interactions were observed between infection status and food availability on any body measurements. An interaction between infection status, food availability, and sex were observed for hind femur length measurements ($F = 6.76$, $df = 1$, $p = 0.014$); no difference was observed between male femur length regardless of infection status or food availability, whereas uninfected female

measurements were less when fed irregularly compared to daily, but no difference was observed for infected females with respect to food availability.

With exception of femur length, *H. convergens* body measurements were significantly greater for females when compared to males (elytra: $F = 9.82$, $df = 1$, $p = 0.004$; pronotum: $F = 11.07$, $df = 1$, $p = 0.002$; head capsule: $F = 5.49$, $df = 1$, $p = 0.026$). Interactions between sex and infection status were significant for hind femur length only ($F = 7.90$, $df = 1$, $p = 0.008$). *H. convergens* larvae that were provided a daily supply of aphids were significantly larger than those fed irregularly (elytra: $F = 39.51$, $df = 1$, $p < 0.001$; pronotum: $F = 60.50$, $df = 1$, $p < 0.001$; femur: $F = 13.23$, $df = 1$, $p < 0.001$; head capsule: $F = 71.70$, $df = 1$, $p < 0.001$; Table 2.5). Pronotum length ($F = 7.46$, $df = 1$, $p = 0.01$) and head capsule width ($F = 4.49$, $df = 1$, $p = 0.042$) were significantly greater when larvae consumed a *T. hippodamiae*-infected egg, compared to those larvae that ate an uninfected egg. Differences were not observed for elytra or hind femur length. For all body parts examined, no significant interaction was observed between infection status and food availability. Interactions between food availability and sex, and those between infection status, food availability and sex were not significant.

2.2.5. Pathogen load

All *A. bipunctata* provided a *V. adaliae*-infected egg as larvae were confirmed to be infected at the end of the trial. For individuals infected with *V. adaliae*, mean spore counts were significantly higher in irregularly fed beetles: 8.33 ± 0.90 spores/120 μm^2 for beetles that were fed daily and 12.38 ± 1.21 spores/120 μm^2 for those fed irregularly ($F = 1.30$, $df = 18$, $p = 0.02$; Fig. 2.2).

At the end of the trial, all *H. convergens* larvae that had eaten a *T. hippodamiae*-infected egg were confirmed as infected. Means spore counts for microsporidia-infected individuals were higher in beetles fed daily (14.00 ± 2.57 spores/120 $\mu m^{2)}$ compared to those fed irregularly (9.70 ± 1.74 spores/120 μm^2); however, the difference was not significant ($F = 1.03$, $df = 18$, $p = 0.18$; Fig. 2.3).

2.4. Discussion

Horizontal transmission of *V. adaliae* in *A. bipunctata* and *T. hippodamiae* in *H. convergens* was 100%, and this was consistent with the transmission results from previous studies of these

microsporidia on various coccinellid hosts (Joudrey & Bjørnson, 2007; Saito & Bjørnson, 2008; Steele & Bjørnson, 2012). Although microsporidian spores were not detected in two individuals (one *A. bipunctata* and one *H. convergens*) that had eaten microsporidia-infected eggs, these larvae died within the initial 48-h of the trial, likely before an infection could become established. Conversely, spores were detected in all individuals that completed development and infection was achieved after larvae consumed one microsporidia-infected egg. Cannibalism is considered part of the normal coccinellid foraging behaviour and, because cannibalism rates tend to increase as food supplies become depleted (Dixon, 2000c), transmission of microsporidian pathogens is likely to increase among coccinellid populations if prey becomes scarce or absent.

2.4.1 Larval development

Microsporidia are expected to lower host fitness because they are obligate pathogens that depend on host energy for growth and survival (Keeling & Fast, 2002). *A. bipunctata* larvae that ate *V. adaliae*-infected eggs took significantly longer to develop than did larvae that ate uninfected eggs. In a previous study, *V. adaliae*-infected *A. bipunctata* larvae took 18.07 days to develop, representing a delay in development of about one day when compared to uninfected beetles (Steele & Bjørnson, 2012). This delay in development keeps larvae susceptible to cannibalism and predation for an extended period.

A. bipunctata larvae that were provided an irregular diet also took significantly longer to develop than those that were provided a daily supply of aphids (Table 2.2). Depending on the type of prey consumed, *A. bipunctata* reared on reduced or suboptimal prey undergo delayed development, have lower fecundity, reduced egg-hatch, lower adult weight and occasionally higher mortality rates (Bonte et al., 2010; De Clercq et al., 2005; Jalali et al., 2009; Schüder et al., 2004; Ware et al., 2009). Despite these negative effects, *A. bipunctata* have good survival rates when prey are deficient, and there is no significant impact on adult longevity when larvae undergo temporary starvation during development (Schüder et al., 2004; Ware et al., 2009). When unable to find adequate prey in nature, adult *A. bipunctata* compensate through supplementary feeding on flower pollen and this reduces or eliminates the negative impacts caused by an otherwise insufficient diet (De Clercq et al., 2005).

An irregular diet is expected to intensify the negative effects of microsporidioses because suboptimal diets have been shown to increase pathogen susceptibility and virulence. For

example, when the reduviid, *Triatoma infestans* Klug is exposed to the trypanosome *Blastocrithidia triatomae*, *T. infestans* nymphs that are temporarily starved are more susceptible to the pathogen, take longer to develop, and have higher mortality rates (Schaub, 1990). For *A. bipunctata* observed in this study, individuals provided an irregular diet took significantly longer to develop than those that were given an ample supply of aphids daily. Larval development was further delayed for individuals that ate a microsporidia-infected egg and were then provided an irregular diet (Table 2.2).

H. convergens larvae that were provided an irregular diet also took significantly longer to develop than those that were given a daily supply of aphids (Table 2.3). Delays in larval development, associated with an irregular diet, seem to be an adaptation for survival. *H. convergens* provided a suboptimal or artificial diet have an increase in reproductive diapause, reduced fecundity, development delays, lower adult weights and higher mortality (Cottrell and Tillman, 2017; Hussein & Hagen, 1991; Racioppi et al., 1981; Wipperfürth et al., 1987). Michaud and Qureshi (2005) suggest that reproductive diapause is an important adaptation for improving *H. convergens* survival during the summer in western Kansas (USA) when aphids are scarce, and females tend to forgo diapause if they have continuous access to high quality prey. Rankin and Rankin (1980) suggest that starvation functions as an important signal to trigger migratory behavior in *H. convergens*, and this acts as a natural means to offset poor nutrition and low food availability.

One would expect the combined effects of irregular diet and infection to cause a further delay in larval development for *H. convergens*, as was observed for *A. bipunctata*, but this was not the case. Starvation decreases the susceptibility of *H. convergens* to the bacterial pathogen, *Pseudomonas fluorescens* (James & Lighthart, 1992). *H. convergens* larvae fed a steady supply of aphids are more susceptible to *P. fluorescens* than those provided water only, and this stress is thought to activate anti-infection immune responses in *H. convergens*. Pathogen susceptibility increases at elevated temperatures, and this suggests that stress factors can have a synergistic effect on *H. convergens* when it is subjected to a weak pathogen. However, it is important to note that pathogens, even those that infect related hosts, can cause unpredictable effects. For example, in the case of *A. bipunctata*, *V. adaliae* has no effect on fecundity (Steele & Bjørnson, 2012), whereas *T. hippodamiae* reduces the fecundity of *H. convergens* (Joudrey & Bjørnson, 2007;

Saito & Bjørnson, 2008). With this in mind, one cannot make generalizations regarding the effects caused by *V. adaliae* or *T. hippodamiae* in combination with other stress mechanisms.

2.4.2 Larval mortality and adult sex ratio

Both microsporidia and inadequate nutrition are known to independently increase mortality in many insects, including coccinellids (Dixon, 2000a; Tanada & Kaya, 1993). Mortality may be difficult to interpret in studies when the consumption of coccinellid eggs serve as the method of infection, because some coccinellid eggs contain species-specific alkaloids that may be toxic to developing beetle larvae (Cottrell, 2004; Omkar & Gupta al., 2004). Larval mortality in this study did not differ between treatment groups, even though mortality values were quite variable (Tables 2.2 and 2.3). For both *A. bipunctata* and *H. convergens*, however, sample sizes for uninfected larvae that were fed irregularly were low (n = 13 and 12, respectively), and this may explain why a statistical difference was not observed for mortality.

2.4.3. Alkaloids

Adaline content was significantly higher for *A. bipunctata* infected with *V. adaliae* than for uninfected beetles, suggesting that adaline content in the reflex fluid is influenced by infection. Adaline content also differed significantly with respect to food availability. For both uninfected and infected beetles, relative percent of adaline in the reflex-fluid was significantly higher in beetles fed daily compared to those fed irregularly. Under laboratory conditions, larvae have been shown to compensate when prey is scarce by prolonging their development period, increasing prey exploitation efficiencies, and by maximizing prey-biomass conversion efficiencies (Schüder et al., 2004). An additional delay in development was observed for beetles that had been fed irregularly during this study, and this delay could be responsible for the lower alkaloid content observed.

Interestingly, a significant interaction between infection status and food availability was observed on relative percent adaline in reflex-fluid (see Fig. 2.1). For both groups (fed daily and fed irregularly), individuals that were infected with *V. adaliae* produced more adaline in their reflex-fluid than did uninfected individuals. However, the difference in adaline production between uninfected and *V. adaliae*-infected beetles is larger for those fed irregularly (a difference of 18.83 mean relative percent) compared to those fed daily (a difference of 8.30

relative percent). This suggests that limited food availability may drastically reduce adaline production in uninfected *A. bipunctata*, but no such reduction occurs for microsporidia-infected individuals. Infected *A. bipunctata* that are fed irregularly appear able to produce adaline (albeit at a significantly lower level than uninfected beetles), perhaps as an immune response elicited by the pathogen. Insect immune systems are able to recognize foreign organisms through a process of pathogen-associated markers and host recognition receptors, that activates and amplifies antimicrobial activities (Hillyer, 2016). It is possible that *V. adaliae* activates the immune response in *A. bipunctata* and adaline production is amplified as part of the various antimicrobial activities. Uninfected *A. bipunctata* that were fed irregularly produced the lowest levels of adaline, suggesting that uninfected beetles do not use limited food resources for alkaloid production. It is important to note that this data only suggests a relationship between food availability, microsporidiosis and adaline content, but the mechanisms behind the observed higher levels of adaline in *V. adaliae*-infected *A. bipunctata* have yet to be determined.

2.4.4. Adult body measurements

Historically, insect morphometric data has been used for species identification. Morphometric data is also used for geographical distribution comparisons (for native vs. non-native or invasive species) and evaluating food sources for rearing purposes (for mass-rearing or commercial uses). However, up until now, morphometric data has not been used to quantify pathogen effects in lady beetles, particularly those infected with microsporidia. All body measurements recorded during the present study are within the size ranges initially reported by Gordon (1985) for *A. bipunctata* and *H. convergens* (Tables 2.4 and 2.5).

Adult size depends on food quality, food quantity, and temperature during larval growth and development, and rearing lady beetles in different environments can influence adult size (Dixon, 2000b). For *A. bipunctata* fed an irregular diet, there was a decrease in the size of all body parts measured, when compared to those fed daily (Table 2.4). Ware et al. (2009) report that *A. bipunctata* larvae develop more quickly into larger adults when aphid prey is unlimited, whereas larval development is slower and smaller adults are produced when prey is limited. In the case of *H. convergens*, adults fed an irregular diet were smaller than those provided a daily, unlimited diet (Table 2.5). Bukero et al. (2015b) report the largest body measurements (length and breadth) for *H. convergens* larvae, pupae and adults when they are fed fresh aphids, rather

than frozen or dried aphids. These results suggest that nutritional quality plays an important role in determining beetle size and development, as does prey quantity. This trend appears common among coccinellids, with similar results reported for *Harmonia axyridis* and *Coccinella septempunctata* (Bukero et al., 2015a; Dmitriew & Rowe, 2007; Evans, 2009; Rahaman and Aniszewski, 2015).

For many insects, including coccinellids, males are typically smaller than females. This size difference allows the males to spend more time looking for mates and less time looking for food because their smaller body requires less energy for metabolism (Dixon, 2000a). *A. bipunctata* also exhibits sexual dimorphism, whereby females are significantly larger than males (Yasuda & Dixon, 2002). In this study, however, differences in body size between male and female *A. bipunctata* were not observed. For *H. convergens*, elytra length, pronotum width and head capsule width were significantly greater for females than for males. In general, size differences between the sexes tend to increase with larger insect species (Teder & Tammaru, 2005). This could explain why a size difference was observed between male and female *H. convergens*, a larger beetle species, but not *A. bipunctata*, a noticeably smaller species. It is important to note that because *H. convergens* is a larger beetle, any errors in measurements would be less and could account for these observations. Interestingly, the size difference between male and female adults in the current study were not influenced by microsporidiosis or food availability. This is unusual because insects infected with microsporidia typically differ in size and form (Tanada & Kaya, 1993) and the reasoning behind the observations in this study are unknown. However, results may be influenced by the small sample sizes used.

For both *A. bipunctata* and *H. convergens*, some body parts were larger for microsporidia-infected beetles (Tables 2.4 and 2.5) when compared to their uninfected cohorts, and infected larvae also took longer to develop (Tables 2.2 and 2.3). Larval development is under hormonal control, and juvenile hormone (JH) is responsible for maintaining insects in their larval state (Wigglesworth, 1962). For some microsporidia-infected insects, JH interferes with normal development. For example, larval development of the tomato moth, *Lacanobia oleracea* L., is prolonged by the microsporidium *Vairimorpha necatrix*. The pathogen causes a 10-fold increase in JH, which results in larger, late-instar larvae that do not pupate (Down et al., 2008). However, in *V. disparis*-infected gypsy moth, *Lymantria dispar* L., delays in larval development are caused by a significant decrease in the concentration of the JH degrading enzyme, JH-esterase, in the

hemolymph (Karlhofer et al., 2012). In the latter case, the pathogen is thought to impair normal functions of the fat body.

The insect fat body is involved in regulating insect growth (Nijhout, 2003). This involves insulin signaling, a multi-functional process that promotes normal, proportional growth and prevents the overgrowth of body parts (Tennessen & Thummel, 2011). Interestingly, both *V. adaliae* and *T. hippodamiae* infect the fat body of their respective hosts (Bjørnson et al., 2011; Steele & Bjørnson, 2014). Infection of the fat body could impair its normal function, and result in measurable differences in body morphometrics, as were observed in this study.

2.4.5. Pathogen load

For *A. bipunctata* infected with *V. adaliae*, spore counts were significantly higher for beetles fed irregularly than for those fed daily (Fig. 2.2). Suboptimal diets are known to increase pathogen susceptibility and virulence for many insects (Donegan & Lighthart, 1989; Lord, 2010; Schaub, 1990). When *V. adaliae*-infected *A. bipunctata* is provided an irregular diet, larval development is further delayed compared to infected beetles that are provided a regular diet. With respect to alkaloid content and spore loads, limiting food availability doesn't appear to induce any stimulatory immune-responses in *A bipunctata*, as mean spore counts increase and the relative portion of alkaloids in reflex-fluid decreases when lady beetles are fed-irregularly (see Fig. 2.1 and 2.2). These results suggest that an irregular diet, such as those commonly experienced in nature, can increase the negative effects of *V. adaliae* on *A. bipunctata* and decrease the production of adaline in reflex-fluid.

There was no significant difference in pathogen load for *T. hippodamiae*-infected *H. convergens* that were fed daily when compared to those that were fed irregularly (see Fig. 2.3). One would expect the spore counts to increase for beetles fed irregularly, as was observed for *A. bipunctata*; however, infection load appears to decrease in infected *H. convergens* fed irregularly, but the mean spore counts do not differ significantly. Starvation and limited food availability are documented as adaptive stress-induced stimulatory responses in *H. convergens* and may initiate diapause, increasing survival when food is scarce, and function as a signal to initiate adult migration (Rankin & Rankin, 1980; Michaud & Qureshi, 2005). Starvation can also reduce the susceptibility of *H. convergens* to certain pathogens (James & Lighthart, 1992).

The theory of hormesis, or hormetic effect, may explain the reported stress-induced stimulatory responses observed in *H. convergens*. The hormetic theory suggests that exposure to a chemical or environmental stressor, one which is typically associated with damaging effects at high doses, can induce beneficial responses when an organism is exposed at low doses (Martins et al., 2011; Mattson, 2008). Temperature, dietary, and chemical-induced hormesis may be beneficial by inducing an insect immune response and slowing the progression of a particular disease (Cutler, 2013; Cutler & Rix, 2015; Little & Kraaijeveld, 2004; Wojda, 2017). Additionally, pathogens can also act as stressors to induce hormetic effects. For example, the bacterium *Bacillus subtilis* (Ehrenberg) Cohn has a negative impact on reproduction in the common eastern bumble bee, *Bombus impatiens* when ingested (the result of a high-dose), but the same microbe stimulates drone production when exposed topically (a low-dose), likely by inducing a stress response (Ramanaidu & Cutler, 2013). Therefore, based on the results of the current study, it is possible that the pathogen or food availability is inducing hormesis. However, without comparative data on alkaloid content, it is difficult to conclude if stimulatory immune-responses are induced when microsporidia-infected *H. convergens* are fed irregularly.

2.5. Conclusion

As obligate pathogens, microsporidia are known to cause chronic and debilitating disease. One would expect the effects of these pathogens to be intensified when food availability is limited. In populations of heavily-infected invertebrates, more susceptible hosts tend to succumb to food shortages and microsporidiosis, leaving those individuals that are less susceptible to the pathogen to survive (Pulkkinen & Ebert, 2004). This may be the case for *A. bipunctata* because larval development was affected by both microsporidiosis and a limited diet. However, this was not the case for *H. convergens*. Although both *V. adaliae* and *T. hippodamiae* infect coccinellid hosts, one cannot make generalizations regarding the effects caused by these pathogens either alone or in combination with other stress mechanisms.

Table 2.1. Conspecific eggs provided to 24-h old *Adalia bipunctata* and *Hippodamia convergens* larvae and the corresponding feeding schedules following egg consumption.

Treatment	*Adalia bipunctata*	*Hippodamia convergens*	Feeding Schedule
Uninfected / Daily	1 uninfected egg	1 uninfected egg	Daily
Infected / Daily	1 *V. adaliae*-infected egg	1 *T. hippodamiae*-infected egg	Daily
Uninfected / Irregular	1 uninfected egg	1 uninfected egg	Irregular*
Infected / Irregular	1 *V. adaliae*-infected egg	1 *T. hippodamiae*-infected egg	Irregular*

* Larvae fed this 'limited' diet were fed on Days 1, 3, 5, 8, 10, 13, 16, 18, 20, 23, 25, 28, 30 and 33 of the trial.

Table 2.2. Mean development (days), percent larval mortality (%), and adult sex ratios for *Adalia bipunctata* fed uninfected or *Vairimorpha adaliae*-infected eggs as 24h-old larvae and provided a daily or irregular supply of *Myzus persicae* as food.

		Larval Development			Larval Mortality		
Treatment (total *n*)	Sex Ratio (♀ : ♂)	*n*	Mean Days ± SE	*P*- value	*n*	Mortality (%)	*P*- value
Uninfected / Daily (23)	(6:17)	23	17.00 ± 0.20 ac		0	0.00	
Infected / Daily (69)	(26:34)	60	17.92 ± 0.14 bc		9	15.00	
Uninfected / Irregular (20)	(9:4)	13	23.62 ± 1.10 ad		7	35.00	
Infected / Irregular (68)	(13:23)	36	26.06 ± 0.34 bd	0.040*	32	47.06	0.169**

Means with the following letters are significantly different: [a,b] Infection status (uninfected versus microsporidia-infected beetles); [c,d] Food availability (beetles fed daily versus irregularly). *ANOVA ($F = 4.30$, $df = 1$); **Chi-square ($\chi^2 = 1.890$, $df = 1$).

Table 2.3. Mean development (days), percent larval mortality (%), and adult sex ratios for *Hippodamia convergens* fed uninfected or *Tubulinosema hippodamiae*-infected eggs as 24h-old larvae and provided a daily or irregular supply of *Myzus persicae* as food.

		Larval Development			Larval Mortality		
Treatment (total *n*)	Sex Ratio (♀ : ♂)	*n*	Mean Days ± SE	*P*- value	*n*	Mortality (%)	*P*- value
Uninfected / Daily (24)	(12:11)	23	17.57 ± 0.14 ac		1	4.17	
Infected / Daily (71)	(33:33)	66	18.38 ± 0.12 bc		5	7.04	
Uninfected / Irregular (24)	(5:7)	12	27.75 ± 0.65 ad		12	50.00	
Infected / Irregular (70)	(22:23)	45	29.51 ± 0.45 bd	0.228*	25	55.56	0.435**

Means with the following letters are significantly different: [a,b] Infection status (uninfected versus microsporidia-infected beetles); [c,d] Food availability (beetles fed daily versus irregularly). *ANOVA ($F = 1.47$, $df = 1$); **Chi-square ($\chi^2 = 0.608$, $df = 1$).

Table 2.4. Mean body measurements (mm) for *Adalia bipunctata* fed uninfected and *Vairimorpha adaliae*-infected eggs as 24-h old larvae and provided a daily or irregular supply of *Myzus persicae* as food.

		Elytra Length	Pronotum Width	Femur Length	Head Capsule Width
	n	Mean (mm) ± SE	Mean (mm) ± SE	Mean (mm) ± SE	Mean (mm) ± SE
Uninfected / Daily	10	3.55 ± 0.04ab	1.97 ± 0.03b	1.15 ± 0.03bcd	1.07 ± 0.02b
Infected / Daily	10	3.60 ± 0.06ab	1.97 ± 0.05b	1.13 ± 0.01bcd	1.05 ± 0.02b
Uninfected / Irregular	10	3.10 ± 0.05ab	1.70 ± 0.03b	1.04 ± 0.02bcd	0.98 ± 0.01b
Infected /Irregular	10	3.33 ± 0.09ab	1.80 ± 0.05b	1.01 ± 0.02bcd	0.99 ± 0.02b

Means with letters represent the following significant interactions:
[a] Infection status (uninfected vs. microsporidia-infected)
[b] Food availability (larvae fed daily vs. larvae fed irregularly)
[c] Food availability and sex (male vs. female)
[d] Infection status, food availability, and sex
[*] No significant interactions were observed for: infection status vs. food availability, infection status vs. sex, or male vs. female

Table 2.5. Mean body measurements (mm) for *Hippodamia convergens* fed uninfected and *Tubulinosema hippodamiae*-infected eggs as 24-h old larvae and provided a daily or irregular supply of *Myzus persicae* as food.

		Elytra Length	Pronotum Width	Femur Length	Head Capsule Width
	n	Mean (mm) ± SE	Mean (mm) ± SE	Mean (mm) ± SE	Mean (mm) ± SE
Uninfected / Daily	10	4.42 ± 0.04bc	2.16 ± 0.03abc	1.54 ± 0.03bd	1.31 ± 0.01bc
Infected / Daily	10	4.36 ± 0.07bc	2.21 ± 0.03abc	1.55 ± 0.03bd	1.33 ± 0.01bc
Uninfected / Irregular	10	3.95 ± 0.06bc	1.94 ± 0.02abc	1.43 ± 0.02bd	1.20 ± 0.01bc
Infected / Irregular	10	4.08 ± 0.08bc	2.03 ± 0.04abc	1.45 ± 0.03bd	1.23 ± 0.02bc

Means with letters represent the following significant interactions:
[a] Infection status (uninfected vs. microsporidia-infected)
[b] Food availability (larvae fed daily vs. larvae fed irregularly)
[c] Sex (male vs. female)
[d] Infection status and sex
[*] No significant interactions were observed for: infection status vs. food availability, food availability vs. sex, or infection status vs. food availability vs. sex

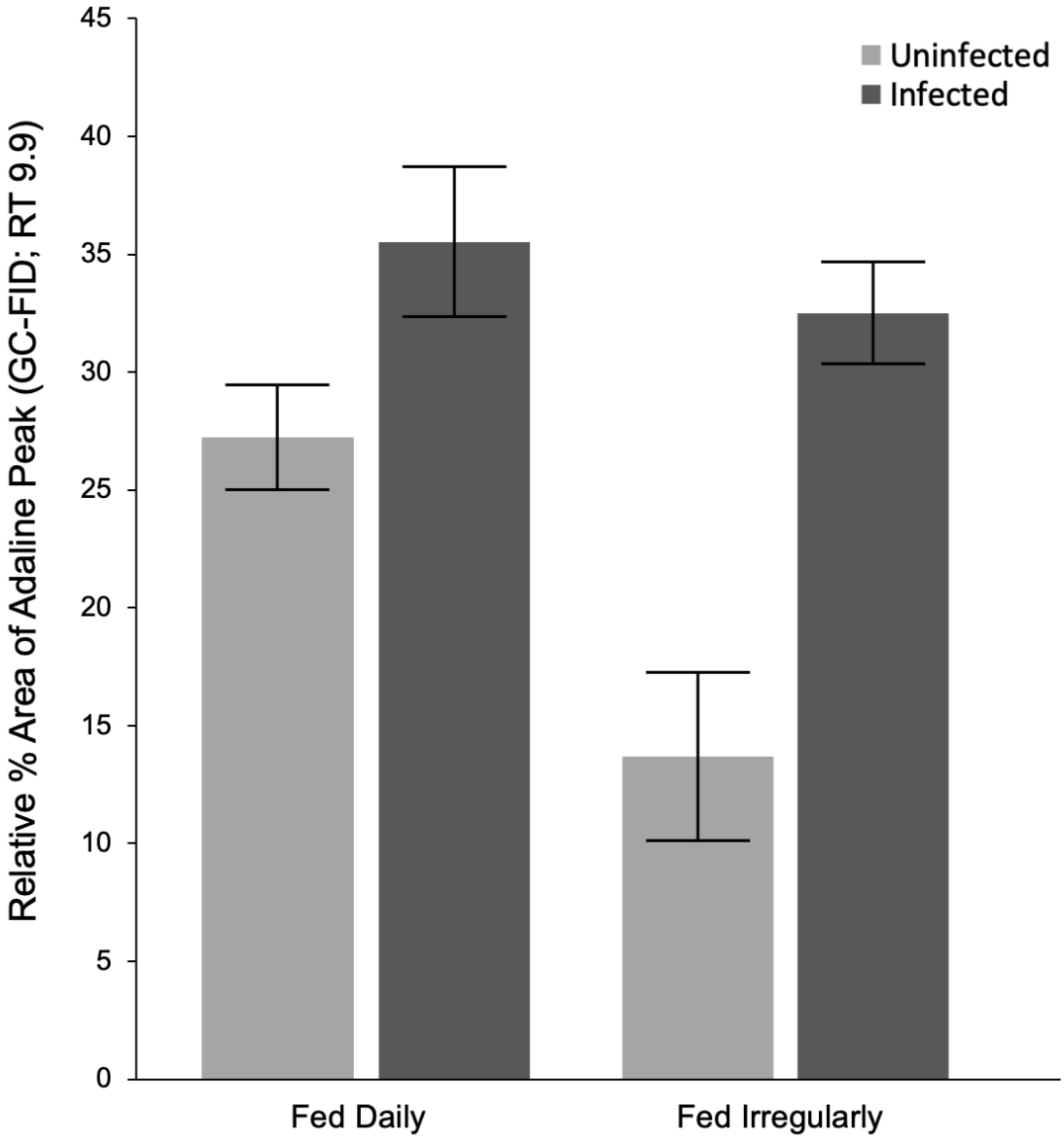


Figure 2.1. Relative percent area of adaline peak (GC-FID; RT: 9.9 min) ± standard error (SE) in reflex-fluid collected from *Adalia bipunctata* adults that ate one uninfected or *Vairimorpha adaliae*-infected egg as 24-h post-hatch larvae, then were fed daily (control) or irregularly (every 2 – 3 days; treatment). A significant interaction between infection status and food availability was observed ($F = 4.53$, $df = 1$, $p = 0.04$).

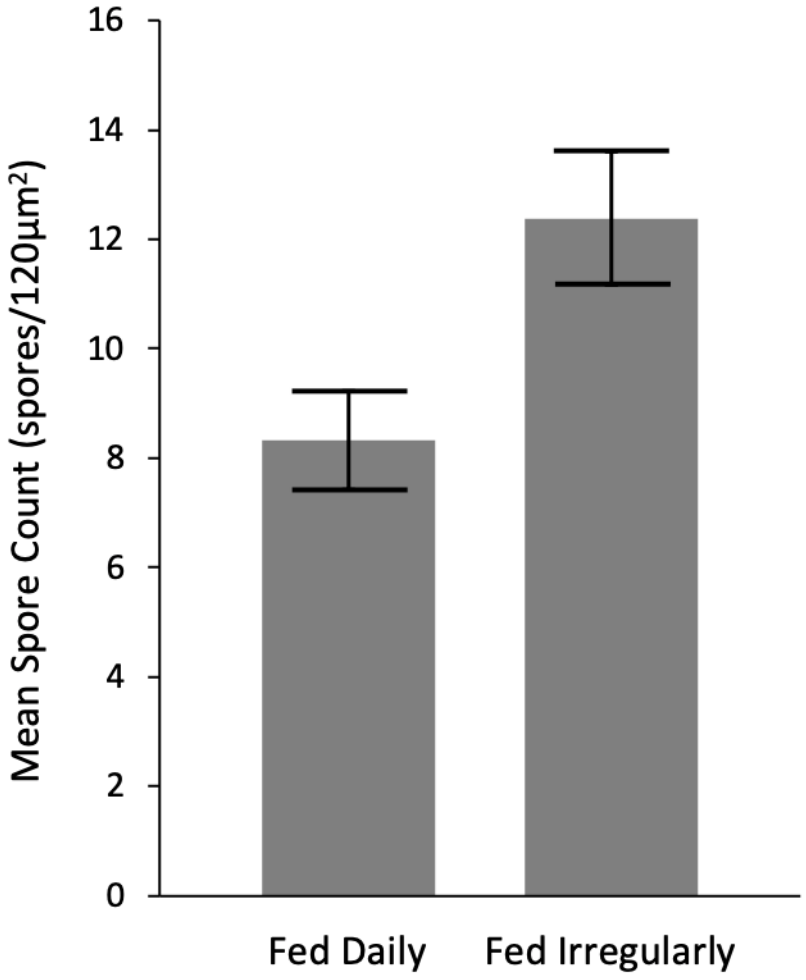


Figure 2.2. Mean spore count (spores/120µm^2) ± standard error (SE) for *Adalia bipunctata* adults that ate one *Vairimorpha adaliae*-infected egg as larvae 24-h post-hatch, then were fed daily (control) or irregularly (every 2 – 3 days; treatment). Mean spore counts differed significantly between control and treatment ($F = 1.30$, $df = 18$, $p = 0.02$).

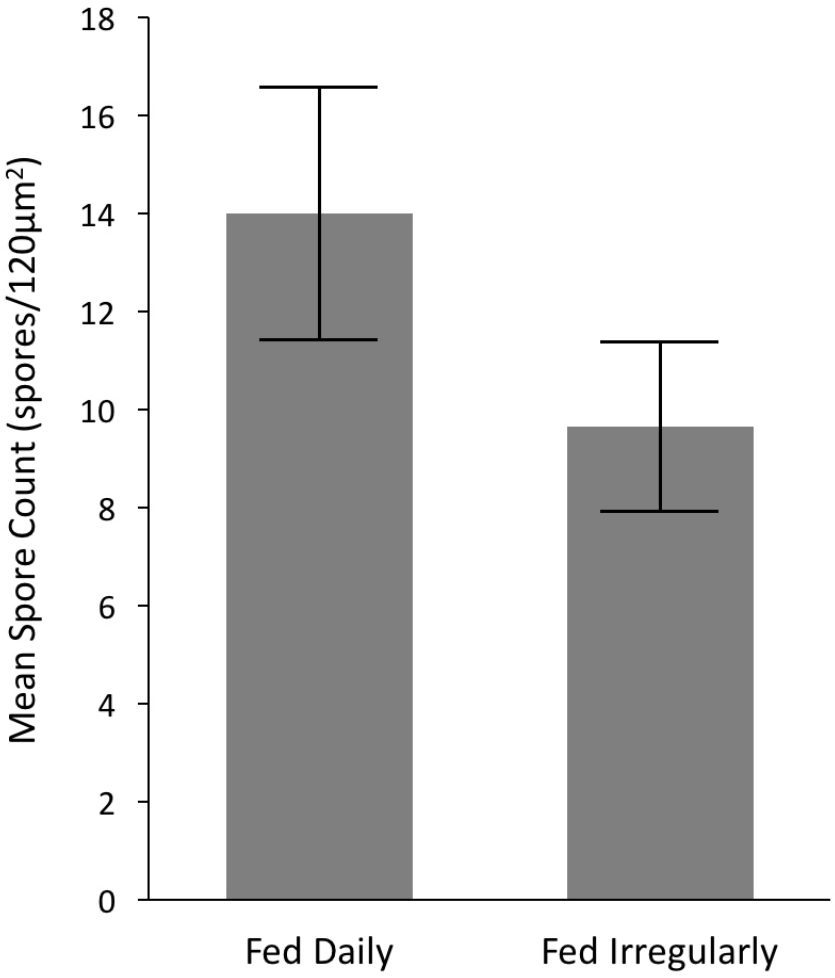


Figure 2.3. Mean spore count (spores/120µm^2) ± standard error (SE) for *Hippodamia convergens* adults that ate one *Tubulinosema hippodamiae*-infected egg as larvae 24-h post-hatch, then were fed daily (control) or irregularly (every 2 – 3 days; treatment). Mean spore counts did not differ significantly between control and treatment ($F = 1.03$, $df = 18$, $p = 0.18$).

3. CHAPTER THREE

Effects of microsporidiosis and temperature on the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae)

3.1. Introduction

Predaceous lady beetles are one of the most recognizable organisms associated with biological pest control (Obrycki & Kring, 1998). These natural enemies are commercially available for controlling a variety of soft-bodied insect pests (Okmar, 2005; Pedigo & Rice, 2006). The two-spotted lady beetle, *Adalia bipunctata* L., has been successfully used as a commercial biological control agent since 1997, after it was released to control rosy apple aphids, *Dysaphis plantaginea* Passerini, on apple trees in Switzerland. *A. bipunctata* was commercialized in 1998 (van Lenteren, 2003; Wyss et al., 1999) and, although these aphidophagous tree-dwelling beetles are native to Europe, Central Asia and North America (Majerus, 1994), they are available for purchase outside of these regions.

Most lady beetles display aposematic colouration that is reinforced by the production and release of species-specific alkaloids through reflex bleeding, a process by which lady beetles secrete a pale-yellow fluid from leg joints and pores when threatened (Hagen, 1962; King & Meinwald, 1996). Reflex-fluid is often toxic to other organisms and acts as a defense mechanism to protect lady beetles from insect and vertebrate predators (Daloze et al., 1995; Majerus, 1994). The alkaloids within the reflex-fluid act as multifunctional defense chemicals, serving as repellents, toxicants, insecticides, surfactants or antimicrobial agents (Dettner, 1987). Alkaloid research in coccinellid hosts has primarily focussed on the antimicrobial properties of these chemicals in the invasive multicolored Asian beetle, *Harmonia axyridis* (Kogel et al., 2012; Röhrich et al., 2012). Two related alkaloids have been described from *A. bipunctata*: the major alkaloid adaline (Tursch et al., 1973) and the minor alkaloid adalinine (Lognay et al., 1996). These alkaloids are present in all life stages of *A. bipunctata* and are known to repel ants (Hill & Renbaum, 1982; Lognay et al., 1996). However, the anti-microbial potential of adaline and adalinine has not been studied, and the potential influence of these alkaloids on insect pathogens is poorly understood.

A. bipunctata is susceptible to *Vairimorpha* (*Nosema*) *adaliae*, a microsporidian pathogen that prolongs larval development, but has no effect on larval mortality, adult fecundity and

longevity, or sex ratios (Steele & Bjørnson, 2012; 2014). Interestingly, the microsporidium *Tubulinosema hippodamiae* from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville, has no effect on larval development *A. bipunctata* (Steele & Bjørnson, 2012), and this raises questions regarding the potential factors that influence the virulence of microsporidiosis in coccinellids. The majority of studies that focus on the effects of microsporidia in coccinellids have been conducted under ideal laboratory conditions. However, stress factors, such as inadequate food sources (those that are scarce or nutritionally deficient), crowding and temperature, are commonly encountered in nature and increase the susceptibility of many insects to disease (James et al., 1998; James & Lighthart, 1992; Steinhaus, 1958).

It is essential for natural enemies to be able to tolerate a wide range of temperatures (Jalali et al., 2010). Thermal extremes can affect mating, diapause, dispersal, ability to locate prey and survival rates, thereby reducing the efficacy of the natural enemy being used (see review by Thomson et al., 2010). *A. bipunctata* tolerate a wide range of temperatures, and studies have shown that development, fecundity and longevity can vary according to temperature. Ideal temperatures for *A. bipunctata* development range from 22 to 25 °C (Ellingsen, 1969; Jalali et al., 2009; Schüder et al., 2004), but these may vary regionally. Beetles reared around 23 °C experience lower mortality rates, increased longevity and fecundity, and increased egg viability when compared to beetles that are reared at lower (19 °C) or higher (27 °C) temperatures (Jalali et al., 2009). Although *A. bipunctata* can complete development over a wide range of constant temperatures (15–30 °C), larvae fail to eclose when temperatures reach 35 °C (Jalali et al., 2010). However, because *A. bipunctata* exhibits polymorphic colouration, with melanic (predominantly black with red spots) and non-melanic (red with black spots) forms (Omkar, 2005), beetles can respond differently to the same temperature (de Jong et al., 1996).

High rearing temperatures are known to reduce microsporidiosis, albeit temporarily, in insects and mites (Becnel & Undeen, 1992; Olsen & Hoy, 2002; Vorontsova et al., 2004). Although temperature extremes are known to reduce *A. bipunctata* fitness, the consequences of temperature stress on microsporidia-infected beetles have not been investigated. Furthermore, the potential impact of temperature on alkaloid production and microsporidiosis in *A. bipunctata* has not been investigated. The objectives of this study were to examine the effects of elevated temperatures on *A. bipunctata* larval development time, adult morphometrics, and alkaloid content (adaline and adalinine), and to assess the development of microsporidiosis (percent

infected and spore counts) when *A. bipunctata* is infected with the microsporidium *Vairimorpha adaliae*.

3.2. Materials and methods

Uninfected and *V. adaliae*-infected *A. bipunctata* used in this trial were obtained from established laboratory stock colonies. These beetles were reared in clear polyethylene cups (120-mL, Canemco-Marivac Inc., QC). Ventilation was provided through a 2.5-cm mesh-covered hole that had been cut in the side of each cup. Stock beetles were maintained on artificial diet (Lacewing and Ladybug Food, Planet Natural, MT) that had been prepared with equal parts honey. Diet was provided as needed and water was provided daily through a moistened cotton wick (Crosstex International, NY).

Uninfected eggs and larvae used in the study originated from eight *A. bipunctata* mating pairs that were isolated from uninfected stock. *V. adaliae*-infected eggs were obtained from another eight microsporidia-infected *A. bipunctata* mating pairs. Beetle mating pairs were maintained in polyethylene cups and fed a combination of green peach aphids (*Myzus persicae* Sulzer), which were reared on nasturtium (*Tropaeolum minus* L., Dwarf Jewel Mixed; Stokes Seed Ltd., ON) and artificial diet. Egg clutches were collected from mating pairs on a daily basis and these were isolated in clean, polyethylene cups. Test larvae used in the study were removed from these cups following hatch. Plants, aphids, larvae and adult beetles were maintained in separate environmental chambers (Sanyo MLR-350H) under controlled conditions (16:8 L:D; 25 °C:20 °C).

The infection status of all individuals (breeding adults, eggs and test larvae) was confirmed by light microscopy. Prior to trial set-up, eggs were randomly selected from each female, smeared on a microscope slide, stained with 5% Giemsa (Sigma-Aldrich, MO) and examined for the presence of microsporidian spores (400x magnification). At the end of the trial, mating beetles and eclosed trial adults were also stained and examined for spores to confirm infection status. Specimens were smeared onto glass microscope slides and allowed to air-dry. Smear preparations were fixed in methanol (10-min), stained in 5% Giemsa (2-h), rinsed in tap water (10-min), and dehydrated using a series of increasing concentrations of ethanol (70%, 3-min; 80%, 3-min; 90%, 3-min; 95%, 3-min; and absolute ethanol, 3-min). Slides were clarified in xylene (10 min) and mounted in Permount (Fisher Scientific).

3.2.1. Larval development time

To determine the effects of temperature on the development of uninfected and *V. adaliae*-infected *A. bipunctata*, test larvae were allocated to six groups based on infection status and temperature as follows: (1) uninfected/25 °C, (2) microsporidia-infected/25 °C, (3) uninfected/27.5 °C, (4) microsporidia-infected/27.5 °C, (5) uninfected/ 30 °C, and (6) microsporidia-infected/30 °C. Uninfected test larvae (24-h old, unfed) used in the trials were individually isolated within Petri dishes (47-mm diameter, Millipore Corp., MA) that had a 3-cm mesh-covered hole in each lid for air circulation. For each of the six treatment groups, six randomly selected individual larvae were isolated daily over 5 d (n = 30 for each group). Larvae were provided one conspecific egg (either uninfected or *V. adaliae*-infected) on a 6-mm filter paper disk that was placed in the center of the Petri dish. Larvae were provided water through a cotton wick, placed into one of three growth chambers kept a constant temperature (25, 27.5 or 30 °C) and given 24-h to consume the egg. Larvae that molted during the initial 24-h period and did not consume their egg were given an additional 24-h to do so before being provided food. Once larvae consumed the egg, they were then provided with an ample supply of aphids and observed daily until they eclosed. Development time (days) and mortality were recorded. After the larvae emerged as adults, they were given water and allowed to rest for 24-h before being sexed. Once completed, the trial was repeated with new mating pairs isolated from stock colonies following the same procedure described herein (n = 60 in total for each control and treatment group). Environmental chambers were rotated with respect to temperate treatment for the replication to compensate for any potential chamber-effects. Individuals were also rotated with respect to placement (shelving) within each chamber for similar reasons. Mean development time (days) was calculated for individuals that completed larval development and emerged as adults. Data was excluded for larvae that did not eat the conspecific egg or died prematurely within 48-h. These larvae may have been injured by siblings prior to isolation, as larvae were starved to ensure egg consumption, or have been injured during the isolation process. A two-way ANOVA with a Tukey's post-hoc test (SPSS Statistics, IMB) was used to compare the duration of larval development between treatments, and to identify any interactions between infection status and/or temperature on larval development.

3.2.2. Larval mortality and adult sex ratio

All individuals (larvae, pupae, adults) that died during the trial were smeared on glass microscope slides, stained with 5% Giemsa, and examined for the presence or absence of microsporidian spores by light microscopy. A $\chi 2$ test of goodness-of-fit (SPSS Statistics, IMB) was used to compare larval mortality between groups. Adult beetles were sexed 24-h after eclosion by examining their terminal, abdominal sternites under a stereomicroscope. A $\chi 2$ test was also used to analyze sex ratio differences.

3.2.3. Alkaloids

At the end of the trial, adult beetles were given artificial diet and water and allowed to rest undisturbed for an additional 24-h after sexing before a sub-sample of 10 beetles (five female and five male) from each group was selected at random to collect alkaloid samples and take body measurements. The 24-h rest period was included to ensure that the body was fully sclerotized before the beetles were handled. Immediately prior to being photographed for microscopic measurements, alkaloid samples were taken. Beetles were held by the abdomen with soft forceps, then softly prodded with a metal probe until a drop of reflex-fluid was released from the tibiofemoral joint. Reflex-fluid was collected with a sterile 2-µl glass capillary micropipette, and immediately following collection, each micropipette was submerged in a separate vial that contained 0.5 ml of methanol. Vials were stored in a freezer (−20 °C) for 6 months until analysis. To confirm the stability of these alkaloids during storage, fresh reflex-fluid samples were collected and analyzed alongside those collected earlier and results from the fresh samples were compared to those that had been stored.

Gas chromatography (GC) was used with mass spectrometry (MS) for alkaloid identification and flame ionization detection (FID) to determine alkaloid concentrations (proportion of adaline relative to other components of reflex-fluid). Samples were analyzed on a Varian 3800 series gas chromatograph (Agilent Technologies, Santa Clara, CA), equipped with a VF-5ms capillary column (30 m × 0.25 mm; CP8944; Agilent J&W GC Columns, Santa Clara, CA) and FID. The GC injection port was set to 240 °C and samples were injected in both split (MS) and splitless (FID) modes with a split ratio of 0.5. Half a microliter of sample (reflex-fluid in MeOH) was injected into the GC using a Varian CP-8400 autosampler. Column temperature was set to 50 °C with an increase of 20 °C per minute and a hold of 4-min. The maximum

temperature was 330 °C with a hold of 5-min. The FID detector was set to 330 °C. Helium was used as a carrier gas at a flow of 1 ml/min. The GC was interfaced with a Varian Saturn 2000 series GC/MS/MS with electron impact ionization (ionization energy of 70 eV; 20–400 *m*/*z* scan range). Adaline was identified by matching mass spectra with those in the literature (Hautier et al., 2008). Adalinine is not detectable with the methods described here. Saturn GC/MS Workstation (version 6.30) software was used to determine the area of all peaks in each sample. The ratio of each adaline peak (presented as relative % area of adaline peak), relative to other components of reflex-fluid (total area of all peaks), was calculated. Methanol peaks were excluded from the calculations so that proportion of adaline was compared to reflex-fluid only. The response of adaline in each sample was expected to be the same. Averages for each treatment were calculated. A two-way ANOVA with a Tukey's post-hoc test (SPSS Statistics, IMB) was used to compare mean relative proportions of adaline between treatments.

3.2.4. Adult body measurements

Body measurements were taken from the same 10 adults that were used for alkaloid analysis (section 2.3). Beetles were photographed with a Zeiss Axio Imager A1 light microscope and attached Zeiss AxioCam MRc digital camera. Body measurements were taken using the AxioVision software (v 4.6.3) measurement tool. Because sclerotized body parts are the most reliable indicators of size (Daly, 1985; Knapp & Knappová, 2013), the width of the pronotum and head capsule, and the length of the elytra and hind-femur were measured. Measurements were taken of each adult from six photographs (n = 6 per body part), and means were calculated for each body part. A two-way ANOVA with a Tukey's post-hoc test (SPSS Statistics, IMB) was used to compare body measurements between groups, and to identify any interactions between body measurements and infection status or temperature.

3.2.5. Spore load

After being photographed for microscopic measurements, the same sub-sample of ten beetles from each treatment was smeared, stained with 5% Giemsa, and examined by light microscopy to confirm infection status and quantify spore load. Five areas of each microscope slide were selected at random to be photographed using a Zeiss Axio Imager A1 light microscope and attached Zeiss AxioCam MRc digital camera. Spores in each image were counted, and mean

spore load per image (100 μm2) was calculated for each beetle. Because the data were not normally distributed, a Kruskal–Wallis test was used to determine difference in spore load with respect to temperature. A $\chi 2$ test (SPSS Statistics, IMB) was used to compare the ratio of uninfected and infected individuals across the three temperatures (25 °C, 27.5 °C, and 30 °C). A Dunn-Bonferroni post-hoc test was used to compare the effect of temperature on infection.

3.3. Results

Adult beetles used to produce uninfected larvae were examined to confirm infection status. Microsporidian spores were not observed in any uninfected *A. bipunctata* adults or their eggs. Conversely, spores were detected in all of the eggs that originated from *V. adaliae*-infected *A. bipunctata* adults.

3.3.1. Larval development time

Mean development times for *A. bipunctata* that consumed an uninfected or infected *A. bipunctata* egg are reported in Table 3.1. Development time was significantly longer for larvae that were reared at 25 °C than those reared at 27.5 °C and 30 °C ($F = 30.27$; $df = 2$; $p < 0.001$). Development for *A. bipunctata* larvae that consumed an *V. adaliae*-infected egg was significantly longer when compared to those that ate one uninfected egg ($F = 17.40$; $df = 1$; $p < 0.001$). A significant interaction between infection status (uninfected vs. microsporidia-infected) and temperature (25 °C, 27.5 °C and 30 °C) was observed, and infected larvae took significantly longer to develop at 25°C than at 27.5°C or 30°C ($F = 9.39$; $df = 2$; $p < 0.001$).

3.3.2. Larval mortality and adult sex ratio

Larval mortality ranged from 1.82% to 10.91%, but did not differ significantly between groups ($\chi 2 = 4.34$, $df = 5$, $p > 0.50$; Table 3.1). For all groups, sex ratios did not differ significantly from 1:1 (♀: ♂) ($\chi 2 = 4.18$, $df = 5$, $p = 0.52$).

3.3.3. Alkaloids

The major alkaloid adaline was identified by comparing mass spectra to those reported in the literature (see Hautier et al., 2008; Yue et al., 1992). Adaline was found at a retention time of 9.9 minutes. There was no interaction between infection status and temperature with respect to the

relative proportion of adaline in *A. bipunctata* reflex-fluid ($F = 0.30$, $df = 2$, $p = 0.74$). Adaline content did not differ significantly between uninfected and microsporidia-infected *A. bipunctata* ($F = 0.44$, $df = 1$, $p = 0.51$). However, the relative proportion of adaline in reflex-fluid increased as temperatures increased (presented as relative % area of adaline peak, Fig. 3.1) and these values differed significantly ($F = 8.21$, $df = 2$, $p = 0.001$). The relative proportions of adaline at 25 °C and 27.5 °C were significantly lower than at 30 °C, ($p = 0.001$ and 0.022, respectively); however, the relative proportion of adaline at 25 °C and 27.5 °C did not differ significantly ($p = 0.46$). The minor alkaloid adalinine was not detectable with the current collection method and GC-MS analysis. Summary data for all adaline peaks, and examples of the typical output data provided by the software, are available in the Appendix.

3.3.4. Adult body measurements

Body measurements of adult *A. bipunctata* did not differ significantly with respect to infection status ($p > 0.05$; Table 3.2). Body part measurements for beetles reared at 25 °C were larger than those reared at 27.5 °C and 30 °C (elytra: $F = 18.42$, $df = 2$, $p < 0.001$; pronotum: $F = 16.20$, $df = 2$, $p < 0.001$; femur: $F = 7.01$, $df = 2$, $p < 0.001$; head capsule: $F = 3.89$, $df = 2$, $p = 0.02$). Female beetles were significantly larger than male beetles (elytra: $F = 50.76$, $df = 1$, $p < 0.001$; pronotum: $F = 8.99$, $df = 1$, $p < 0.001$; femur: $F = 5.07$, $df = 1$, $p = 0.03$; head capsule: $F = 4.54$, $df = 1$, $p = 0.04$). No significant interactions were observed between infection status and temperature, infection status and sex, or between temperature and sex. A significant interaction between infection status, temperature and sex was observed for elytra length and head capsule width only (elytra: $F = 3.10$, $df = 2$, $p = 0.049$; head capsule: $F = 3.06$, $df = 2$, $p = 0.050$).

3.3.5. Spore load

Beetles that ate *V. adaliae*-infected eggs as larvae and were reared at 25 °C were infected at the end of the trial. However, percent infection decreased to 89.3% (50 of 56 larvae) and 53.8% (21 of 39 larvae) for larvae reared at 27.5 °C and 30 °C, respectively, and these values differed significantly ($\chi2 = 38.02$, $df = 2$, $p < 0.001$; Fig. 3.2). Spore counts also decreased significantly as temperatures increased ($\chi2 = 43.03$, $df = 22$, $p < 0.001$; Fig. 3.2). Mean spore counts were 31.11 spores/100 µm at 25 °C. The mean number of spores decreased significantly to 10.46 and

0.94 spores/100 μm2 at 27.5 °C and 30 °C, respectively (25 °C to 27.5 °C, $p = 0.005$; 25 °C to 30 °C, $p < 0.001$; 27.5°C to 30°C, $p = 0.002$).

3.4. Discussion

Horizontal transmission of *V. adaliae* in *A. bipunctata* larvae reared at 25 °C was consistent with results from previous studies (100% transmission at 25 °C; Steele & Bjørnson, 2012, 2019). Although horizontal transmission of *V. adaliae* in *A. bipunctata* was 100% when reared at 25 °C, the number of infected individuals significantly decreased when reared at 27.5 °C and 30 °C. This suggests that the observed decrease in horizontal transmission observed at 27.5 °C and 30 °C is attributed to temperature or increased alkaloid content.

3.4.1. Larval development time

Microsporidia are obligate pathogens that rely on their hosts for resources (Agnew et al., 2003), and delays in development are expected because these pathogens deplete the energy reserves of the host. According to Steele and Bjørnson (2012, 2019), *V. adaliae*-infected larvae take about one day longer to complete development at 25 °C than do uninfected larvae. In the current study, development of *A. bipunctata* larvae that ate a single *V. adaliae*-infected egg was delayed significantly when compared to larvae that consumed an uninfected *A. bipunctata* egg (Table 3.1).

The results from the current study showed that larval development time decreased significantly as temperatures increased. Jalali et al. (2009, 2010) also showed that the development time of *A. bipunctata* larvae decreases significantly when temperatures increase. An increase of 4 °C is enough to shorten larval development by 60 to 80%, and *A. bipunctata* larvae fail to complete development at 35 °C.

A significant interaction between infection status and temperature was observed on larval development during this study (Table 3.1). Infected larvae took significantly longer to develop at 25 °C than at 27.5 °C, 30 °C, and when compared to their uninfected counterparts at the same temperature (Table 3.1). Infected beetles reared at 25 °C had higher spore loads than did those reared at 27.5 °C or 30 °C (Fig. 3.2). It is possible that the energy demands of the pathogen on the host larvae are less at 30 °C than at 25 °C, because higher temperatures have been shown to increase the metabolic rates of insects and shorten their development times (Denlinger & Yocum,

1998; Neven, 2000). When infected *A. bipunctata* larvae were reared at 27.5 °C and 30 °C, fewer individuals became infected (89.3 and 53.8%, respectively) and the pathogen had no effect on larval development.

3.4.2. Larval mortality and adult sex ratio

Both microsporidia and high rearing temperatures are known to increase mortality rates in many insects (Jalali et al., 2010; Neven, 2000; Tanada & Kaya, 1993). Larval mortality in this study was relatively low and did not differ between treatment groups (Table 3.1). Mortality rates were consistent with previous reports for *A. bipunctata* larvae infected with *V. adaliae* (Steele & Bjørnson, 2012; 2019), and in reports that ascertained the optimal rearing temperatures for *A. bipunctata* (Jalali et al., 2009, 2010).

Some microsporidia distort the sex ratios of invertebrates (Dunn et al., 1993; Engelstädter and Hurst, 2009; Ironside et al., 2003). However, the sex ratios of *A. bipunctata* adults in this study were about 1:1 (♀: ♂), regardless of treatment (Table 3.1). This result is consistent with previous reports of sex ratios for both uninfected and *V. adaliae*- infected *A. bipunctata* (Steele & Bjørnson, 2012; 2019).

3.4.3. Alkaloids

Alkaloids are used by lady beetles for chemical defense against predation, but these chemicals also are thought to play a role in the insect immune system. With respect to coccinellids, research has focused on alkaloids from the invasive multicoloured Asian lady beetle, *H. axyridis*, to determine why this beetle is so successful at outcompeting native lady beetle species. The reflex-fluid of *H. axyridis* contains the alkaloid harmonine, that shows broad-spectrum antimicrobial activity against several human pathogens (Kogel et al., 2012; Röhrich et al., 2012). When *A. bipunctata* were infected with the microsporidian pathogen *V. adaliae*, beetles had slightly higher levels of adaline in their reflex-fluid when compared to uninfected beetles, but this difference was not significant. Rearing larvae at 27.5 °C did not influence adaline levels in infected adults. However, beetles reared at 30 °C had significantly higher relative proportions of adaline in their reflex-fluid than did beetles reared at 25 °C or 27.5 °C. Because an increase in temperature tends to increase the metabolic functions of insects (Denlinger & Yocum, 1998), higher rearing temperatures could be responsible for the observed increase in adaline production.

Increases in adaline production may also be a result of a result of a stimulatory immune-response such as hormesis. The theory of hormesis suggests that stressors typically associated with unfavorable consequences for insects when exposed at high levels, such as temperature, pesticides, caloric restriction and hypoxia, may have a beneficial effect when insects experience the same stressor at a much lower level (Cutler et al., 2022). Interestingly, the results suggest that high levels of adaline correspond to decreased percent infection, and those individuals that became infected had low spore counts. These observations may be the result of rearing individuals at 30 °C, the increased adaline in these individuals when reared at 30 °C, or a combination of both. However, when interpreting the results for alkaloid concentrations within reflex-fluid, it is also important to consider individual variation before making any conclusions. de Jong et al. (1991) found significant variation among beetles with respect to adaline concentration and amount of reflex-fluid produced, suggesting internal factors within individuals (such as genetic factors) may determine how much energy is invested into chemical defence. Larger sample sizes would be beneficial to compensate for individual variation. To determine causation for the increased adaline production, and to account for individual variation, further studies are needed.

3.4.4. Adult body measurements

The insect fat body is involved in regulating insect growth (Nijhout, 2003), and because *V. adaliae* infects the fat body of *A. bipunctata* (Steele & Bjørnson, 2014), one would expect to observe differences in adult body measurements because of infection. In a previous study, *V. adaliae*-infected *A. bipunctata* had larger elytra when compared to uninfected adults (Steele & Bjørnson, 2019). Interestingly, none of the body parts measured in our study differed significantly when individuals were compared solely by their infection status (Table 3.2).

It is difficult to make confident conclusions regarding the effects of microsporidiosis on body morphometrics because horizontal transmission of *V. adaliae* in *A. bipunctata* was less than 100% for individuals reared at 27.5 and 30 °C, and mean spore counts decreased as temperatures increased (Fig. 3.2). However, there were no significant interactions observed between infection status or temperature on body measurements. Although the body measurements between uninfected and infected beetles did not differ significantly, mean elytra length and pronotum width of uninfected adults reared at 25 °C were noticeably smaller than those from

microsporidia-infected adults. These same body parts are nearly identical in size for uninfected and microsporidia-infected adults reared at 30 °C. These results suggest that *V. adaliae* had no effect on body measurements *A. bipunctata*.

Terrestrial insects that develop at higher temperatures typically are smaller in size because higher temperatures hasten growth and shorten development times (see Kingsolver & Huey, 2008). This was the case for *A. bipunctata*. Elytra length, pronotum width, femur length, and head capsule width were all smaller for adults that were reared at 27.5 and 30 °C (Table 3.2). Although measurements from sclerotized body parts are the most reliable indicators of size in insects, body weight has been used to make size comparisons among lady beetles in the majority of previous studies. Because body weight exhibits a positive linear relationship with size measurements for coccinellids (Rogers et al., 1977), measurement trends observed in the current study can be compared to the trends in body weight reported in the literature. When *A. bipunctata* is reared in laboratory conditions at three different temperatures (19 °C, 23 °C and 27 °C), weight increases as temperatures rise from 19 to 23 °C; after this point body weight decrease as temperatures rise to 27 °C (Mills, 1979). This trend is well documented for other coccinellids. For example, the ideal rearing temperature of the convergent lady beetle, *H. convergens*, with respect to size, is 22 °C. At this temperature, this species has higher pupal and adult weights, and adults have longer wings when compared to conspecifics reared at 18, 26, and 30 °C (Rodriguez-Saona & Miller, 1999). In the case of the seven-spotted lady beetle, *Coccinella septempunctata*, and the multicolored Asian lady beetle, *H. axyridis*, temperature determines the body weight and fat body content in adults (Krengel et al., 2012). However, it is important to note that size isn't the only metric to be considered when determining ideal temperatures for coccinellids.

Sexual size dimorphism is common among coccinellids, and *A. bipunctata* females are significantly larger than males (Yasuda & Dixon, 2002). In our study, mean body measurements were significantly larger for females than for males across all temperatures, regardless of infection (Table 3.1). Infected males had larger elytra and head capsules than did uninfected males at 25 °C, but no difference was observed at 30 °C. Males, which are smaller with less fat body than females, may be more affected by a larger pathogen load in this organ at 25 °C compared to 30 °C. Heat can further amplify sexual dimorphism for some coccinellids, whereby size differences between the sexes are much more pronounced at higher temperatures (Krengel et

al., 2012). In the current study, the average body size for male and female *A. bipunctata* was not exaggerated when examined at 25 °C to 30 °C. This is consistent with observations made by Mills (1981) for *A. bipunctata*.

3.4.5. Spore load

The number of infected individuals and their average spore counts decreased dramatically as rearing temperatures increased (Fig. 3.2). These observations may be the result of higher rearing temperatures, the increased adaline in individuals reared at higher temperatures, or a combination of both. However, the benefits of rearing arthropods at high temperatures to control microbial infections and parasitoids is well documented in the literature and many insects have an adaptive behavioural fever response that they use to combat infections (Heinrich, 1993).

Lethal temperatures for microsporidian pathogens vary depending on their host. When infected hosts are exposed to temperatures that exceed 32 °C, microsporidian spore loads may be reduced (Becnel & Undeen, 1992; Olsen & Hoy, 2002) or eliminated (Vorontsova et al., 2004). Yet, other species of microsporidia are able to tolerate extreme temperatures (60 °C) and remain viable (Fenoy et al., 2009). The effect of temperature on microsporidia that infect lady beetles has not been reported; however, infection rates of the entomopathogenic fungus *Beauveria bassianna* in *H. convergens* decreases as temperature increases from 15 °C to 35 °C (James et al., 1998). Results from the current study suggest that temperature limits for *V. adaliae* may also fall within this range. It is important to note that only a subset of constant temperatures were examined during this study. *A. bipunctata* would experience fluctuating temperatures in nature and be able to respond to high temperatures by dispersing to cooler areas.

3.5. Conclusion

In nature, lady beetles experience a wide range of environmental temperatures that pathogens must tolerate in order to be successful and proliferate. According to the literature, high rearing temperatures are known to reduce microsporidiosis in numerous insect species and are a common technique used to reduce infections in mass-rearings. Based on the results of the current study, temperature can have a mitigatory effect on *V. adaliae* in two-spotted lady beetles, with similar temperatures ranges as other microsporidian species in similar hosts. Beetles reared at 27 °C and 30 °C had lower spore loads and percent infection compared to those reared at 25 °C.

Additionally, beetles reared at 30 °C had significantly higher relative proportions of adaline in their reflex-fluid than did beetles reared at 25 °C or 27.5 °C. Further studies are needed to determine the mechanisms behind these observations (is it due to an increase in metabolic processes from increased temperatures, or a result of a stimulatory immune-response such as hormesis?) and the relationship between alkaloids and infections (the insect immune system). It would also be worthy to investigate the relationship between colder temperature-extremes and microsporidia in lady beetles to determine if the trends observed in this study are reflected or reversed.

Table 3.1. Mean development (days), larval mortality (%), and adult sex ratios for *Adalia bipunctata* that ate one uninfected or *Vairimorpha adaliae*-infected egg as 24h-old larvae, then reared at 25°C, 27.5°C or 30°C.

		Larval Development			Larval Mortality		
Treatment (total *n*)	Sex Ratio (♀ : ♂)	*n*	Mean Days ± SE	*P*- value	*n*	Mortality (%)	*P*- value
Uninfected 25°C (57)	(29:25)	54	13.94 ± 0.08		3	5.26	
Infected 25°C (55)	(25:24)	49	15.12 ± 0.13		6	10.91	
Uninfected 27.5°C (55)	(25:29)	54	13.85 ± 0.17		1	1.82	
Infected 27.5°C (56)	(24:28)	52	14.06 ± 0.14		4	7.14	
Uninfected 30°C (53)	(23:25)	48	13.35 ± 0.18		5	9.43	
Infected 30°C (39)	(13:23)	36	13.42 ± 0.11	< 0.001*	3	7.69	0.501**

*Two-way ANOVA ($F = 9.39$, $df = 2$); **Chi-square ($\chi^2 = 4.34$, $df = 5$).

Table 3.2. Mean body measurements (mm) for *Adalia bipunctata* adults that ate one uninfected or *Vairimorpha adaliae*-infected egg as 24h-old larvae, then reared at 25°C, 27.5°C or 30°C.

		Elytra Length	Pronotum Width	Femur Length	Head Capsule Width
	n	Mean (mm) ± SE	Mean (mm) ± SE	Mean (mm) ± SE	Mean (mm) ± SE
Uninfected 25°C	20	3.65 ± 0.07*	2.04 ± 0.03	1.07 ± 0.02	1.10 ± 0.01*
Infected 25°C	20	3.73 ± 0.05*	2.07 ± 0.02	1.07 ± 0.01	1.11 ± 0.01*
Uninfected 27.5°C	20	3.60 ± 0.04*	1.97 ± 0.03	1.06 ± 0.02	1.09 ± 0.01*
Infected 27.5°C	20	3.55 ± 0.04*	1.97 ± 0.02	1.02 ± 0.02	1.07 ± 0.01*
Uninfected 30°C	20	3.45 ± 0.05*	1.93 ± 0.02	1.03 ± 0.01	1.09 ± 0.01*
Infected 30°C	20	3.43 ± 0.05*	1.93 ± 0.02	0.99 ± 0.01	1.08 ± 0.01*
25°C	40	3.67 ± 0.04a	2.05 ± 0.02a	1.07 ± 0.01a	1.11 ± 0.01a
27.5°C	40	3.59 ± 0.03b	1.97 ± 0.02b	1.05 ± 0.01b	1.08 ± 0.01b
30°C	40	3.44 ± 0.04c	1.93 ± 0.02c	1.01 ± 0.01c	1.08 ± 0.01c
Uninfected	60	3.57 ± 0.03	1.98 ± 0.02	1.05 ± 0.01	1.09 ± 0.01
Infected	60	3.58 ± 0.03	1.99 ± 0.01	1.03 ± 0.01	1.09 ± 0.01
Females	60	3.69 ± 0.03d	2.01 ± 0.02d	1.05 ± 0.01d	1.10 ± 0.01d
Males	60	3.45 ± 0.03e	1.96 ± 0.01e	1.03 ± 0.01b	1.08 ± 0.01b

Means with different letters represent a significant difference with respect to (1) temperature (25°C, 27.5°C or 30°C) or (2) sex (female vs. male). * Interaction between infection status (+/-), temperature and sex for elytra length and head capsule width.

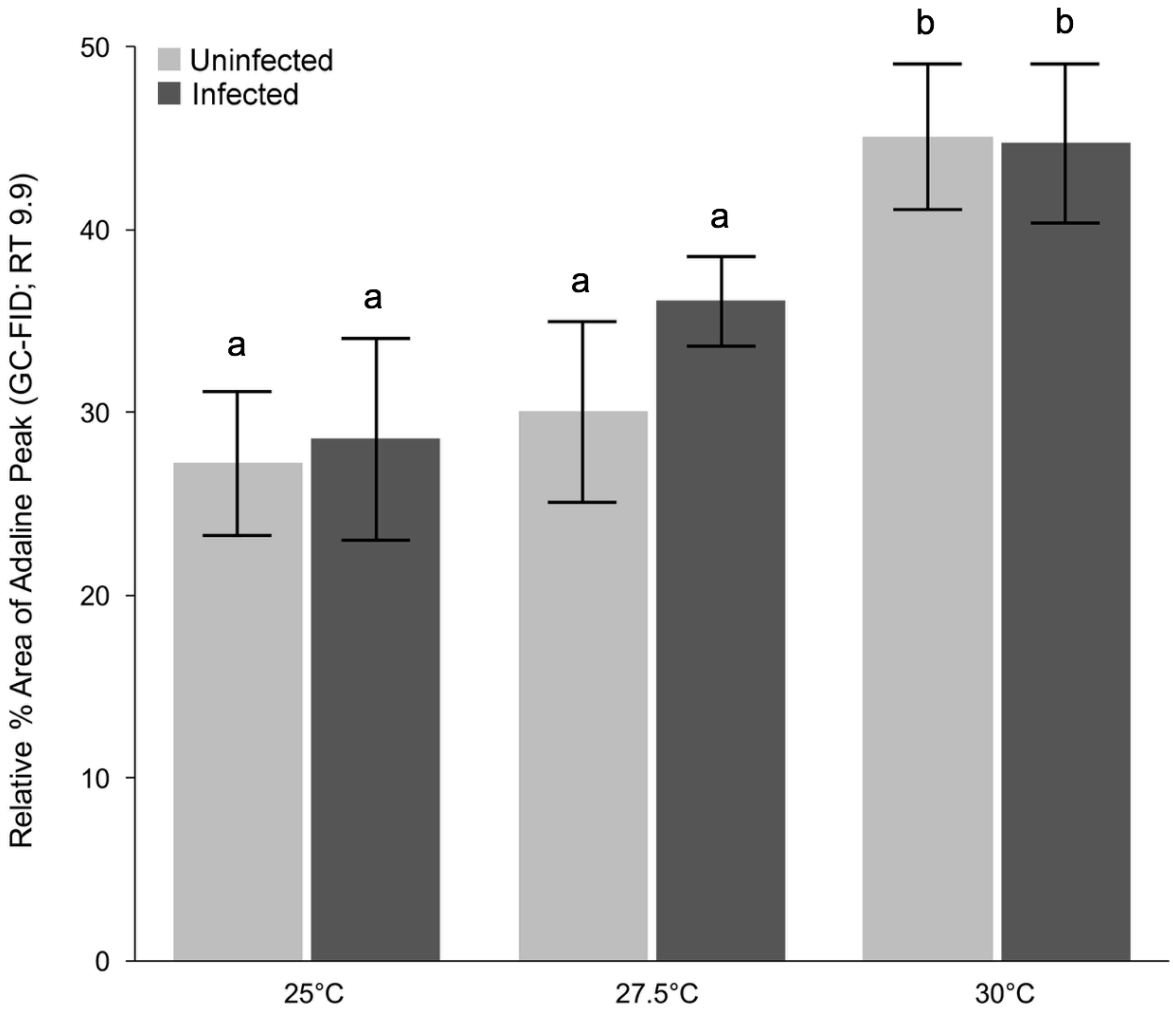


Figure 3.1. Relative percent area of adaline peak (GC-FID; RT: 9.9 min) ± standard error (SE) in reflex-fluid collected from *Adalia bipunctata* adults that ate one uninfected or *Vairimorpha adaliae*-infected egg as 24h-old larvae, then reared at 25°C, 27.5°C or 30°C. Means with different letters represent a significant difference ($F = 8.207$, $df = 2$, $p = 0.001$).

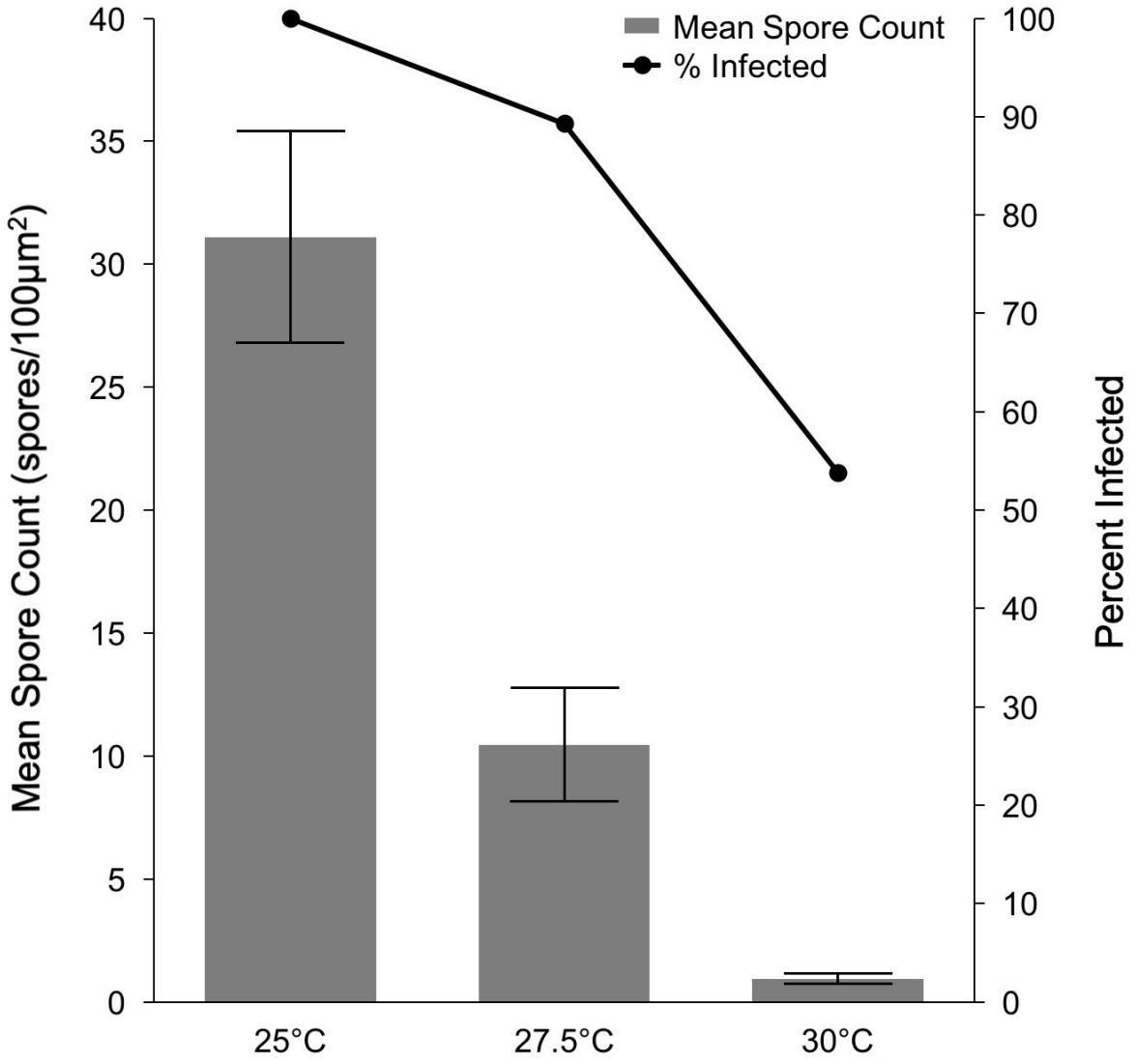


Figure 3.2. Mean spore count (spores/100µm^2) ± standard error (SE) and percent infection for *Adalia bipunctata* adults that ate one *Vairimorpha adaliae*-infected egg as 24h-old larvae, then reared at 25°C, 27.5°C or 30°C. Percent infected and mean spore counts were significantly different between all temperatures ($\chi^2 = 38.02$, $df = 2$, $p < 0.001$ and $\chi^2 = 43.03$, $df = 2$, $p < 0.001$, respectively.

4. CHAPTER FOUR

Alkaloid content in microsporidia-infected *Adalia bipunctata* (Coleoptera: Coccinellidae) life stages, and pathogen spore load in adults after exposure to physical stress

4.1. Introduction

The two-spotted lady beetle, *Adalia bipunctata* L., is a beneficial insect that is commercially available for biological pest control. *A. bipunctata*, like many other coccinellids, displays warning colouration of vibrant reds and oranges with contrasting black spots. This aposematic colouration warns potential predators of lady beetle unpalatability and toxicity that is due, in part, to the production of noxious alkaloid compounds (Ceryngier et al., 2012; Daloze et al., 1995; King & Meinwald, 1996; Laurent et al., 2005; Omkar & Pervez, 2016). Alkaloids are a large group of heterocyclic (typically) nitrogen-containing organic compounds of biological origin that exhibit pharmacological activity (Aniszewski, 2015). Lady beetles produce alkaloids as secondary metabolites in the insect fat body from simple fatty acid precursors (Gronquist & Schroeder, 2010; Laurent et al., 2002). Two alkaloids have been identified from *A. bipunctata*: the major alkaloid adaline (Tursch et al., 1973) and the minor alkaloid adalinine (Lognay et al., 1996). Alkaloids are thought to be present throughout the entire lifecycle, have been shown to repel other insects, and are suspected to play a role in the insect immune system (Lognay et al., 1996; Marples, 1993; Verheggen et al., 2017). However, the quantity of alkaloids at each life stage, and the relationship (if any) between these defence chemicals and chronic infections, such as those caused by microsporidia, has yet to be established.

Microsporidia are spore-forming obligate intracellular parasites that take advantage of host resources for their own replication before they are transmitted to new cells or a new susceptible host (Agnew et al., 2003; Keeling & Fast, 2002; Vavra & Larsson, 2014). These pathogens, despite being completely dependent on their hosts for survival (Keeling & Fast, 2002; Keeling, 2009), often cause sublethal chronic disease (see Bjørnson & Oi, 2014). *Vairimorpha* (*Nosema*) *adaliae*, a microsporidium described from *A. bipunctata*, delays larval development under controlled laboratory conditions, but has no effect on mortality or adult fecundity and longevity (Steele & Bjørnson, 2012; 2014). When *A. bipunctata* is infected with *V. adaliae*, beetles produce more alkaloids (measured as a higher relative proportion of adaline in the reflex-

fluid) when compared to uninfected beetles (Steele et al., 2020a). However, it is not known if this trend is consistent throughout all stages of development.

Stress factors, such as poor nutrition, over-crowding, and temperature extremes, have been shown to increase an insect's susceptibility to disease (James et al., 1998; James & Lighthart, 1992; Steinhaus, 1958). However, other stressors, such as physical agitation, can reduce an insect's susceptibility to disease. When larvae of the greater wax moth, *Galleria mellonella*, are exposed to physical stress (shaking), susceptibility to the fungal pathogen *Candida albicans* is significantly reduced (Mowlds, 2008). Shaking resulted in elevated haemocyte numbers and antimicrobial peptides in the haemolymph and is believed to prime *G. mellonella* immune responses (Mowlds, 2008). Irregular food supply increases spore loads in *A. bipunctata* infected with *V. adaliae*, but this is not the case when the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville is infected with the microsporidium *Tubulinosema hippodamiae* (Steele & Bjørnson, 2019). Elevated rearing temperatures (above 25 °C) seem to mitigate the development of *V. adaliae* in *A. bipunctata* (Steele et al., 2020b). These inconsistent observations raise questions regarding the virulence of different species of microsporidia in insects, the susceptibility of their various hosts, and the factors that may influence infection. Previous studies documented the effects of temperature and food availability on the development of *A. bipunctata* on adult alkaloid production and microsporidiosis, but the impact of physical stress on alkaloid production in uninfected and infected *A. bipunctata* has not been investigated. The objectives of this study were to (1) determine the effects of a microsporidiosis on the production of alkaloid (adaline) defense compounds during the development of *A. bipunctata*, and (2) to evaluate the combined effects of physical stress (shaking agitation) and infection (*V. adaliae*) on adult beetles (relative alkaloid content and infection load).

4.2. Materials & Methods

Uninfected and *Vairimorpha adaliae*-infected (hereafter referred to as infected) *Adalia bipunctata* were obtained from established laboratory stock colonies. Stock beetles were reared in clear polyethylene cups (120-mL, Canemco-Marivac Inc., QC) with a 2.5-cm mesh-covered hole for ventilation. Beetles were maintained on artificial diet (Lacewing and Ladybug Food, Planet Natural, MT; prepared with equal parts honey) that was provided as needed. Water was provided daily through a moistened cotton wick (Crosstex International, NY).

Eggs and larvae used in the study were obtained from mating pairs: eight uninfected *A. bipunctata* mating pairs isolated from uninfected stock and eight microsporidia-infected *A. bipunctata* mating pairs isolated from infected stock. Mating pairs were kept in individual polyethylene cups and fed a diet of green peach aphids (*Myzus persicae* Sulzer) that were reared on nasturtium (*Tropaeolum minus* L., Dwarf Jewel Mixed; Stokes Seed Ltd., ON), supplemented with artificial diet. Eggs were collected daily. Plants, aphids, larvae and beetles were maintained in separate environmental chambers (Sanyo MLR-350H) under controlled conditions (16:8 L:D; 25°C:20°C).

The infection status of individuals used in this study (parents, eggs and test larvae) was verified by examining sibling eggs using light microscopy. Prior to trial set-up, randomly selected eggs from each mating pair were smeared on a microscope slide, stained with 5% Giemsa (Sigma-Aldrich, MO) and examined for the presence of microsporidian spores (400x magnification). At the end of the trial, mating pairs and all test individuals were examined for spores to confirm infection status.

4.2.1. Larval development & sample collection

To compare alkaloid content (the relative proportion of adaline in hemolymph; RP adaline) throughout larval development, uninfected and infected *A. bipunctata* were reared in isolation within individual Petri dishes (47-mm diameter, Millipore Corp., MA) that had a 3-cm mesh-covered hole in the lid for ventilation. Individuals were analyzed for adaline content at all stages of development: egg, first-instar (L1), second-instar (L2), third-instar (L3), fourth-instar (L4), pupa and adult (see Figure 4.1, experimental design). Over each day for 5 days, and for both uninfected and infected treatments, two eggs and two first-instar larvae were immediately prepared for alkaloid analysis (n = 10 for each stage). These were dissected in individual vials containing 2 mL methanol. Another 21 first-instar larvae were isolated in individual petri dishes daily (n = 10 for each developmental stages [50 total] and an additional n = 6 for the adult stress trial [30 total]). Larvae were provided water and an ample supply of aphids. Each larva was observed daily until they were analyzed for alkaloids at the appropriate developmental stage or had eclosed as an adult. Alkaloid samples were collected during the development trial (second-instar through adult) through reflex-bleeding, whereby each individual was gently prodded with a blunt metal probe until it released fluid from between the segments of the exoskeleton and/or

tibiofemoral joints. The reflex-fluid was collected with 2 μL glass capillary micropipettes, which were immediately submerged in methanol (2 mL) within individual glass vials. Samples were stored at -20°C until ready for analysis (4 – 16 weeks). Eggs, first-instar larvae and pupae were sampled directly because reflex-fluid could not be collected from these stages. Those larvae that developed fully and emerged as adults were given water and left undisturbed for 24h. Adults were sexed by examining their terminal, abdominal sternites under a stereomicroscope. Development time and mortality were recorded. The trial was repeated with new mating parents isolated from stock colonies.

All individuals (larvae, pupae, adults) that died during the trial were smeared, stained with 5% Giemsa, and examined for the presence or absence of microsporidian spores by light microscopy. Larvae that died with 72-h of trial set-up were excluded from data analysis. Mean development time (days) was calculated for individuals that completed larval development and emerged as adults. A two-tailed t-test was used to determine significance in the duration of larval development between uninfected and microsporidia-infected beetles. A χ^2 test (SPSS Statistics, IMB) was used to compare larval mortality between groups. A χ^2 test was also used to compare sex ratios.

4.2.2. Alkaloid content through development

Samples collected during the larval development trial were analyzed for alkaloid content with a Varian 3800 series gas chromatograph (Agilent Technologies, Santa Clara, CA), equipped with a VF-5ms capillary column (30 m X 0.25 mm; CP8944; Agilent J&W GC Columns, Santa Clara, CA) and flame ionization detection (FID). The GC injection port was set to 240°C and samples were injected in splitless mode for both MS and FID. A 0.5 microliter sample (hemolymph or reflex fluid in MeOH) was injected into the GC using a Varian CP-8400 autosampler. Column temperature was set to 50°C with an increase of 20°C per minute and a hold of 4-min. The maximum temperature was 330°C with a hold of 5-min. The FID detector was set to 330°C. Helium was used as a carrier gas at a flow of 1 mL/min. The GC was interfaced with a Varian Saturn 2000 series GC/MS/MS with electron impact ionization (ionization energy of 70 eV; 20 – 400 m/z scan range). Because these compounds are not commercially or readily available in purified form, adaline was visually identified by matching mass spectra with those in the literature (Hautier et al., 2008). Adalinine was not detectable with the current methods used.

Saturn GC MS Workstation (version 6.30) software was used to calculate the relative proportion area of the adaline peak in each sample (relative proportion of adaline to hemolymph/reflex-fluid, excluding methanol; RP adaline). Averages for each group were calculated and two-sample t-tests (SPSS Statistics, IMB) were used to compare relative alkaloid content between uninfected and infected individuals at each stage.

4.2.3. Adult agitation stress: Alkaloid content and infection load

To examine the effects of physical stress (shaking/agitation) on alkaloid production and spore load, a subsample of adults from the development trial were selected for use in an adult stress trial (n = 30 uninfected and n = 30 microsporidia-infected beetles). These adults were divided into the following three experimental groups (n = 10 per group): 1) control (no shaking/agitation); 2) alternate shaking/agitation (every other day); 3) daily shaking/agitation. Individuals in the control group were observed daily for 5 days and provided ample food and water. Those in the treatment groups were exposed to shaking (roughly 126 shakes per minute for 3 minutes by hand, within individual dishes) at different intervals (every other day or daily) for 5 days. These agitation methods used were meant to stimulate a stress response and represent mild disturbances beetles may experience in nature, such as wind blowing through the foliage they are inhabiting. Preliminary practice trials were conducted using stock beetles to ensure shaking rates did not injure the beetles or induce reflex-bleeding (confirmed visually and olfactorily). Contents of each dish were removed prior to shaking for the same reason. At the end of day five (seven days post-eclosion), reflex-fluid was collected from each individual for alkaloid analysis before each was dissected on a microscope slide. Reflex-fluid was analyzed using the same methodology (GC-MS/FID) and statistical analyses (two sample t-tests) as described in section 2.2. Microscope slides were stained with 5% Giemsa and examined by light microscopy to confirm infection status and quantify spore load. Five areas of each microscope slide were selected at random for examination with a Zeiss Axio Imager A1 light microscope equipped with an ocular grid reticle at 100x objective (oil-immersion, 1000x magnification). Mean spore load per unit area (120 μm^2) was calculated for each beetle. Because spore data was not normally distributed and originated from small sample sizes, the Kruskal–Wallis H test with a Dunn's Multiple comparisons post-hoc test (SPSS Statistics, IMB) was used to compare mean spore counts between groups.

4.3. Results

All individuals used in the trial were confirmed to have the correct infection status. Microsporidian spores were not detected in parent beetles used to obtain uninfected eggs, and all test individuals (eggs, larvae and adults) remained uninfected at the end of the trial. Conversely, spores were detected in all of the eggs that originated from infected *A. bipunctata* parents and infection of all trial individuals was confirmed post-mortem.

4.3.1. Larval development

Mean development for uninfected and infected *A. bipunctata* was 16.65 days ± 0.16 and 18.75 days ± 0.19 (SE) days, respectively. Development time of infected larvae was significantly longer than for uninfected larvae ($F = 3.251$; $df = 203$; $p < 0.001$). Larval mortality was around 15.00% for uninfected and infected larvae, but mortality did not differ significantly between groups ($\chi 2 = 0.001$, $df = 1$, $p = 0.978$; Table 4.1). Sex ratios of uninfected and infected beetles did not differ significantly from one another ($p > 0.05$).

4.3.2. Alkaloid content through development

The major alkaloid adaline was identified by comparing mass spectra to those reported in the literature and was found at a predetermined retention time of 9.9 minutes. RP adaline for uninfected *A. bipunctata*, gradually increases from egg until L4 and peaks at the pupal stage (Figure 4.2). Additionally, RP adaline for *V. adaliae*-infected *A. bipunctata* increases from egg to pupa, and peaks at the adult stage. Adalinine was not detectable with the current methods used, most likely because adalinine is present in low concentrations (adaline concentrations are 10X greater than that of adalinine; Lognay et al., 1996).

A significant difference in RP adaline between uninfected and infected individuals was observed for all stages except for L1 and L2 (Figure 4.2). Uninfected eggs had significantly more RP adaline than did infected eggs, whereas L3, L4, pupal, and adult stages of uninfected individuals had significantly less RP adaline when compared to infected counterparts. Because the method of alkaloid collection differed between early and late developmental stages, the progression of RP adaline throughout development was not analyzed. However, there was an increasing trend in relative alkaloid content for both uninfected and infected *A. bipunctata* as individuals completed development (Figure 4.2). Infected newly emerged adults (48-hrs old) had

higher RP adaline compared to 7-day old adults (27.26 ± 3.18 SE and 10.15 ± 2.61 SE, respectively; $F = 0.82$, $df = 38$, $p < 0.01$), but there was no difference in RP adaline between newly-eclosed and older uninfected adults (12.21 ± 1.39 SE and 6.08 ± 0.47 SE, respectively; $p > 0.05$). RP adaline did not differ significantly between sexes, regardless of infection status ($p > 0.05$). Summary data for all adaline peaks, and examples of the typical output data provided by the software, are available in the Appendix.

4.3.3. Adult agitation stress: Alkaloid content and spore load

RP adaline for uninfected adults that were not shaken (control), and those were subjected to alternate shaking and daily shaking were 6.08 ± 0.47 SE, 10.73 ± 1.68 SE and 9.00 ± 1.54 SE percent area peak, respectively (Figure 4.3). Conversely, RP adaline for infected adults were 10.15 ± 2.61 SE, 6.31 ± 0.90 SE and 9.86 ± 1.82 SE percent area peak, respectively. When uninfected and infected adults were exposed to varying levels of agitation, the RP adaline differed significantly only for individuals that were shaken during alternate days. Uninfected adults that were shaken on alternate days had significantly higher RP adaline when compared to infected adults ($F = 6.54$, $df = 38$, $p = 0.026$; Figure 4.3). When comparing treatment groups within the same infection status were compared, significant differences were not observed between the different levels of agitation ($p > 0.05$).

Average spore counts for individuals that were not shaken (control), those that were shaken on alternate days, and those shaken daily were 20.07 ± 1.88 SE, 26.72 ± 3.54 SE, and 41.79 ± 4.35 SE spores per 120 μm^2, respectively (Figure 4.4). Infection load differed significantly for beetles that had been agitated ($H = 16.86$, $df = 2$, $p < 0.001$). Although there was no difference in mean spore load between the control (no shaking) and alternate shaking groups ($Z = 16.86$, $p = 0.304$), adults that were exposed to daily shaking had significantly higher mean spore counts than those of the control (exposed to no shaking; $Z = 16.86$, $p < 0.001$) and alternate shaking ($Z = 16.86$, $p = 0.003$).

4.4. Discussion

The infection status of all individuals was confirmed at the end of the trial. Vertical transmission of *V. adaliae* in *A. bipunctata* was 100%. These results are consistent those reported in previous studies (see Table 4.2).

4.4.1 Larval development, mortality & sex ratios

According to results from previous studies, when *V. adaliae* is transmitted horizontally to uninfected *A. bipunctata* through egg consumption, larvae take about one day longer to complete development than do larvae that consume an uninfected egg (Table 4.2). Individuals in the current study obtained their infection vertically, from *V. adaliae*-infected parents. These larvae took about 2 days longer to complete development than did uninfected *A. bipunctata* (Table 4.1). When infected horizontally, larvae experience a period of developmental time without exposure to the pathogen. However, when infected vertically, individuals are exposed to the pathogen at the onset of development. These different modes of transmission could be responsible for the additional delay in larval development observed in the current study compared to previous reports.

Some species of microsporidia significantly increase mortality rates and distort sex ratios in insect hosts (Bandi et al., 2001; Bjørnson & Oi, 2014; Vilcinskas et al., 2013). However, this is not reported for *A. bipunctata* infected with *V. adaliae* (Tables 4.1 and 4.2).

4.4.2. Alkaloid content through development

Alkaloid content has been studied in only a few coccinellids, and these compounds have been quantified throughout development in only two species: the Mexican bean beetle *Epilachna varivestis*, and the South American ladybird beetle *Epilachna paenulata* (Camarano et al., 2006; Goetz et al., 1986; Proksch et al., 1993). Adaline and adalinine, the two alkaloids present in *A. bipunctata*, were thought to be present in all life stages (Lognay et al., 1996; Pasteels et al., 1973) but have not been previously quantified. The current study quantified adaline (relative proportion) in each developmental stage of uninfected and *V. adaliae*-infected *A. bipunctata*. In general, RP adaline increased during development from egg to adult, regardless of infection status (Figure 4.2). Alkaloid production is strongly correlated with body weight in adult *A. bipunctata* (Holloway et al., 1993). Results of the current study indicate that smaller, mobile larvae, which are able to flee from predators, produce lower levels of adaline, whereas the larger stationary pupae, which are more vulnerable to predation, produce higher levels of adaline.

Uninfected *A. bipunctata* produced higher proportions of adaline during early stages of development (egg to L2) when compared to infected individuals. However, as development progressed beyond L2, adaline content for infected beetles significantly surpassed that of

uninfected beetles (Figure 4.2). Additionally, RP adaline peaked at the pupal stage for uninfected *A. bipunctata* but adaline continued to increase in infected adults. Steele et al. (2020a) observed that *V. adaliae*-infected *A. bipunctata* adults produce significantly more adaline (RP in reflex-fluid) than did uninfected beetles. This suggests that microsporidiosis may be responsible for the observed increase in adaline content. In the current study, adaline content was significantly greater in infected *A. bipunctata* during the later stages of development only (L3 to adult). The data shows that adaline production increases with development, as would the progression of the infection. Although infected adults have a significantly higher level of adaline than uninfected adults, the infected eggs produced by the former have significantly lower levels of adaline when compared to uninfected eggs. The reason why high levels of adaline are not passed from infected female to egg remains unknown. The results suggest that the infected egg is less protected by the major alkaloid adaline, and this may make infected eggs less toxic to other coccinellids. Cannibalism is common among lady beetles, being particularly prevalent in *A. bipunctata* (Dimetry, 1974; Omkar & Pervez, 2005). Because adaline provides chemical protection against predation by non-conspecifics (Ware et al., 2008; 2009), low levels of adaline would be less likely to deter coccinellid predators and could ultimately help ensure that the pathogen is effectively transmitted to other susceptible hosts.

Newly emerged *V. adaliae*-infected adults (48-h) had significantly higher RP adaline in their reflex-fluid than did seven-day old adults (see Figures 4.2 and 4.3), whereas uninfected adults had similar proportions regardless of age. These results show that uninfected beetles maintained a consistent level of adaline production over 5 days, but infected beetles did not. *V. adaliae* infects the insect fat body (Steele & Bjørnson, 2014), which is responsible for many metabolic functions, including the production of alkaloids (Laurent et al., 2002). Infection of the fat body by *V. adaliae* may disrupt metabolic activities and could explain the observed differences in RP adaline among adults.

In the current study, only adaline from *A. bipunctata* was examined. Adalinine was not detectable with the current methods used. In previous studies, adalinine was successfully isolated from samples that consisted of hundreds of adults that had been pooled together for analysis (Lognay et al., 1996). In the current study, each alkaloid sample was extracted from an individual, and as a result, the concentration of adalinine within each sample was likely too low to be detectable. Future studies should include the quantification of adalinine to provide further

insight regarding the protective role of all alkaloids in *A. bipunctata*. For example, Goetz et al. (1986) examined the alkaloid euphococcinine in developmental stages of the Mexican bean beetle, *Epilachna varivestis* Mulsant, and found that eggs were devoid of euphococcinine, despite it being the major alkaloid of the adults. Authors suggest that additional alkaloids may be produced by *E. varivestis* that were not examined in their study. Proksch et al. (1993) later confirmed eight other alkaloids in *E. varivestis* that differed with respect to presence and ratios at each developmental stage. Results showed that the eggs, larvae and pupae of *E. varivestsis* accumulate only pyrrolidine alkaloids, which are gradually replaced by piperidine alkaloids as development progresses. Similar observations were found for the South American ladybird beetle *Epilachna paenulata* Germar. Four alkaloids identified in *E. paenulata* change in proportion throughout development: eggs contain high amounts of one alkaloid, whereas adults contain a mixture of up to four different alkaloids (Camarano et al., 2006). It is possible that adalinine is only present in some developmental stages. Additionally, there may be other alkaloids present in *A. bipunctata* that have yet to be characterized.

It is also important to note that considerable individual variation in alkaloid production is documented for *A. bipunctata* (Dixon, 2000; de Jong et al., 1991), and this is reflected by the standard errors reported in the current study (see Figure 4.3). de Jong et al. (1991) suggests that certain individual factors, such as genetic variation, determine individual alkaloid production. Holloway et al. (1993) examined the potential genetic costs and trade-offs that are associated with chemical defence in *A. bipunctata*. Following their investigation, the authors discuss the complexity of such a study but offer no definitive conclusions. Interestingly, individual variation in alkaloid production is also documented in other lady beetles, including *Harmonia axyridis* (Pallas), *C. septempunctata* L., and *Hippodamia convergens* (Holloway et al., 1991, 1993; Kajita et al., 2010).

4.4.3. Adult agitation stress: Alkaloid content and infection load

Overall, increasing exposure to physical agitation had no significant impact on alkaloid production for uninfected or infected individuals (Figure 4.3). In previous studies where adaline production in *A. bipunctata* was examined, elevated temperatures significantly increase RP adaline in beetle reflex-fluid, whereas irregular food availability decreases RP adaline content (Steele et al., 2020a; 2020b). Because stress is known to influence metabolic functions in many

insects (Janković-Hladni, 2018), one would expect alkaloid production to be affected by exposure to physical agitation. However, the agitation trial used in the current study may not have been long enough in duration to detect any differences in alkaloid production. The rate of physical agitation (shaking) used in this study may have been insufficient and did not induce stress responses. Also, the results may have been confounded by the young age of the adults used. For *E. varivestis*, pupae and newly emerged adults possess similar alkaloid types and quantities, but the adults do not fully adopt their characteristic alkaloid profile until 10 to 14 days post eclosion (Proksch et al., 1993). In the current study, both uninfected and infected *A. bipunctata* adults were producing high RP adaline that was similar to what was produced by the pupal stages at the beginning of the agitation trials (see Figure 4.2).

Microsporidiosis impacts host alkaloid production when individuals are subjected to agitation. In the current study, uninfected beetles produced significantly more adaline than infected individuals when they were subjected to alternate shaking (Figure 4.3). Differences were not observed between uninfected and infected beetles in the control or daily shaking groups. When beetles were exposed to shaking every other day (alternate shaking), uninfected individuals maintained consistent adaline levels, but infected beetles did not. The unpredictability of alternate shaking may be more stressful and challenging for individuals to overcome than predictable daily shaking. However, caution should be taken when interpreting these results because of the considerable individual variation observed in this study, as noted in the standard errors, and small sample sizes (n = 20 per group). Similar results were reported in a previous study with considerable individual variation, where no difference in RP adaline was found between uninfected and infected *A. bipunctata* adults (Steele et al., 2020b).

Stress factors are known to increase the susceptibility of many insects to disease (James et al., 1998; James & Lighthart, 1992; Steinhaus, 1958). Nutritional stress, induced by an irregular food supply during larval development, increases pathogen load (mean spore counts) in *V. adaliae*-infected *A. bipunctata,* compared to adults fed daily (Steele et al., 2020a). These higher spore counts correspond to high RP adaline in reflex-fluid. Heat stress, caused by rearing *A. bipunctata* at temperatures above 25 °C has a mitigatory effect on *V. adaliae*. A decrease in transmission and pathogen load in adults reared at 30 °C corresponded to an increase in RP adaline content (Steele et al., 2020b). In the current study, average spore counts were significantly higher in *A. bipunctata* that were exposed to daily agitation; however, these

differences in pathogen load are not correlated with any significant changes in alkaloid production (Figures 4.3 and 4.4). This suggests that infected beetles are unable to simultaneously combat the pathogen and produce adaline while undergoing physical stress. Conversely, prior exposure to a sublethal dose of a stressor (such as shaking) can increase the immune response and provide an insect with some resistance to a subsequent infection (Browne et al., 2014; Sheehan et al., 2020). For example, when greater wax moth larvae (*Galleria mellonella* L.) are exposed to physical stress, they become less susceptible to the pathogenic fungi *Candida albicans* and *Aspergillus fumigatus* when infected 24-hours after shaking (Browne et al., 2014; Mowlds et al., 2008). These observations support a hormetic principle that exposure to a low-dose environmental agent will result in a stimulatory or beneficial effect, whereas a high-dose agent will result in an inhibitory or toxic effect (Calabrese & Baldwin, 2002; Cutler, 2013; Cutler et al., 2022; Mattson, 2008). Since the current study exposed individuals to agitation after establishing an infection, no conclusions can be made in support of hormesis in *A. bipunctata* exposed to mild, short-term agitation stress. However, examining hormesis with respect to microsporidian infections in beneficial lady beetles is an area worthy of future investigation.

4.5. Conclusion

Alkaloid production (RP) increases throughout *A. bipunctata* development, as each developmental stage faces environmental pressures and predation risks. When uninfected, RP adaline peaked at the pupal stage, a stage when *A. bipunctata* are highly vulnerable to predation. When infected with *V. adaliae*, RP adaline peaked in the adult stage. Uninfected eggs contained significantly more adaline than infected eggs, but RP adaline was similar for L1 and L2 stages. From L3 onwards, infected individuals contained significantly more RP adaline than did their uninfected counterparts. When adults were exposed to physical agitation, adaline production was not affected by the agitation frequency for beetles with the same infection status. However, uninfected beetles had higher RP adaline than did infected beetles only when individuals were exposed to alternate shaking. Average spore counts increased significantly when adults were exposed to more frequent amounts of shaking, suggesting that physical agitation can increase *A. bipunctata* susceptibility to *V. adaliae*.

Table 4.1. Mean development (days), percent larval mortality (%), and adult sex ratios for uninfected and *Vairimorpha adaliae*-infected *Adalia bipunctata*.

		Larval Development			Larval Mortality		
Treatment (total *n*)	Sex Ratio (♀ : ♂)	*n*	Mean Days ± SE	*P*- value	*n*	Mortality (%)	*P*- value
Uninfected (103)	(55:48)	103	16.65 ± 0.16		18	14.88	
Infected (102)	(48:54)	102	18.75 ± 0.19	< 0.001*	18	15.00	0.98**

*Two-sample T-test (F = 3.251, *df* = 203); **Chi-square (χ^2 = 0.001, *df* = 1).

Table 4.2. Reported effects of *Vairimorpha adaliae* on *Adalia bipunctata* exposed to different stress factors.

Conditions	Treatment	Infection (+/-)	Development (Days ± SE)	Mortality (%)	Sex Ratios (♀:♂)	Transmission (% Infected)	Spore Load (/120 µm^2 ± SE)	Study
Multiple Infections [*V. adaliae* + *T. hippodamiae*]	Control	Uninfected	16.75 ± 0.18	8.33	20:34	N/A	Not reported	Steele & Bjørnson, 2012
	One pathogen	Infected	18.07 ± 0.18	4.84	27:31	100	Not reported	
	Two pathogens	Infected	18.29 ± 0.10	14.49	35:24	100	Not reported	
Food Availability	Fed Daily	Uninfected	17.00 ± 0.20	0	6:17	N/A	N/A	Steele & Bjørnson, 2019; Steele et al., 2020a
	Fed Irregularly	Uninfected	23.62 ± 1.10	35.00	9:4	N/A	N/A	
	Fed Daily	Infected	17.92 ± 0.14	15.00	26:34	100	8.33 ± 0.9	
	Fed Irregularly	Infected	26.06 ± 0.34	47.06	13:23	100	12.38 ± 1.2	
Temperature	25 °C	Uninfected	13.94 ± 0.08	5.26	29:25	N/A	N/A	Steele et al., 2020b
	27.5 °C	Uninfected	13.85 ± 0.17	1.82	25:29	N/A	N/A	
	30 °C	Uninfected	13.35 ± 0.18	9.43	23:25	N/A	N/A	
	25 °C	Infected	15.12 ± 0.13	10.91	25:24	100	31.11 ± 4.3	
	27.5 °C	Infected	14.06 ± 0.14	7.14	24:28	89.3	10.46 ± 2.3	
	30 °C	Infected	13.42 ± 0.11	7.69	13:23	53.8	0.94 ± 0.2	

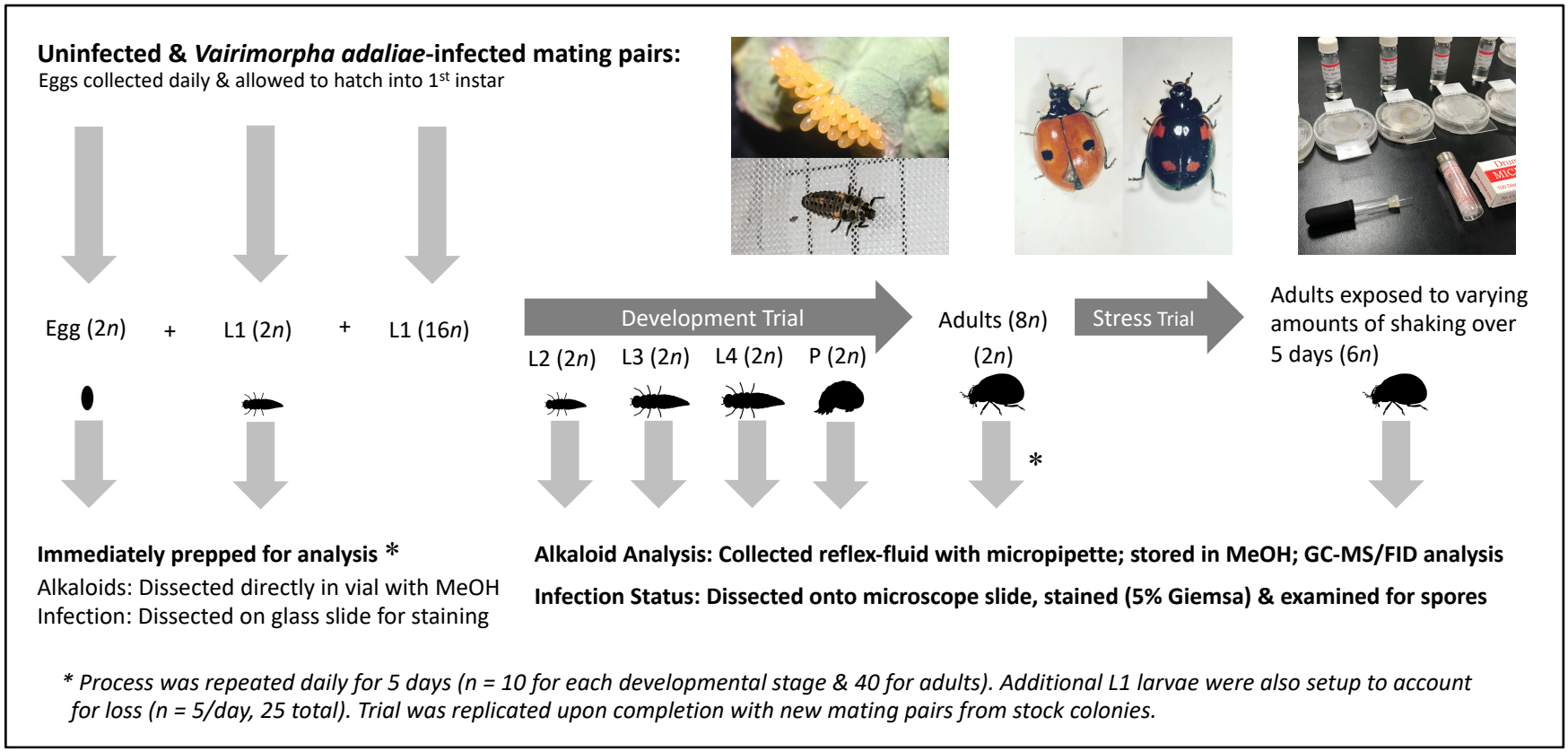


Figure 4.1. Alkaloid and infection load sample collection for uninfected and *Vairimorpha adaliae*-infected *Adalia bipunctata* throughout development and during adult physical stress trials.

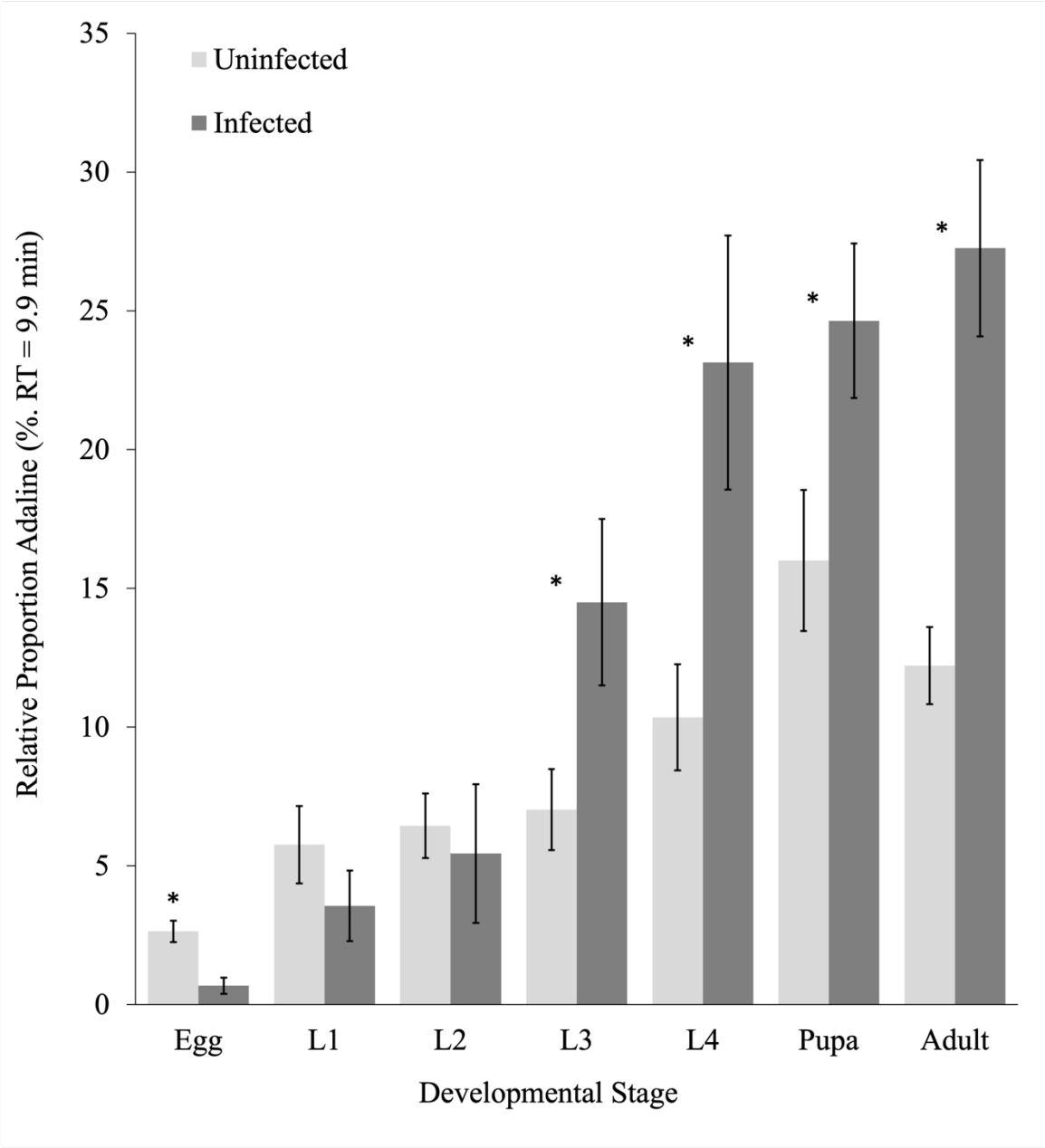


Figure 4.2. Relative percent area of adaline peak (GC-FID; RT: 9.9 min) ± standard error (SE) in hemolymph collected from uninfected and *Vairimorpha adaliae*-infected *Adalia bipunctata* at each developmental stage. Means with an asterisk (*) represent a significant difference between uninfected and infected at that stage: Egg (2.64 ± 0.39 vs. 0.68 ± 0.29*, $F = 2.01$, $df = 38$, $p < 0.001$); L1 (5.76 ± 1.40 vs. 3.56 ± 1.27, $F = 0.07$, $df = 38$, $p = 0.250$); L2 (6.45 ±1.16 vs. 5.44 ± 2.50, $F = 2.80$, $df = 38$, $p = 0.717$); L3 (7.03 ± 1.46 vs 14.50 ± 3.0*, $F = 13.74$, $df = 38$, $p = 0.031$); L4 (10.35 ± 1.91 vs, 23.14 ± 4.57*, $F = 36.16$, $df = 38$, $p = 0.014$); Pupa (16.01 ± 2.54 vs. 24.60 ± 2.78*, $F = 0.05$, $df = 38$, $p = 0.028$); Adult: (12.21 ± 1.39 vs. 27.26 ± 3.17*, $F = 7.49$, $df = 38$, $p < 0.001$).

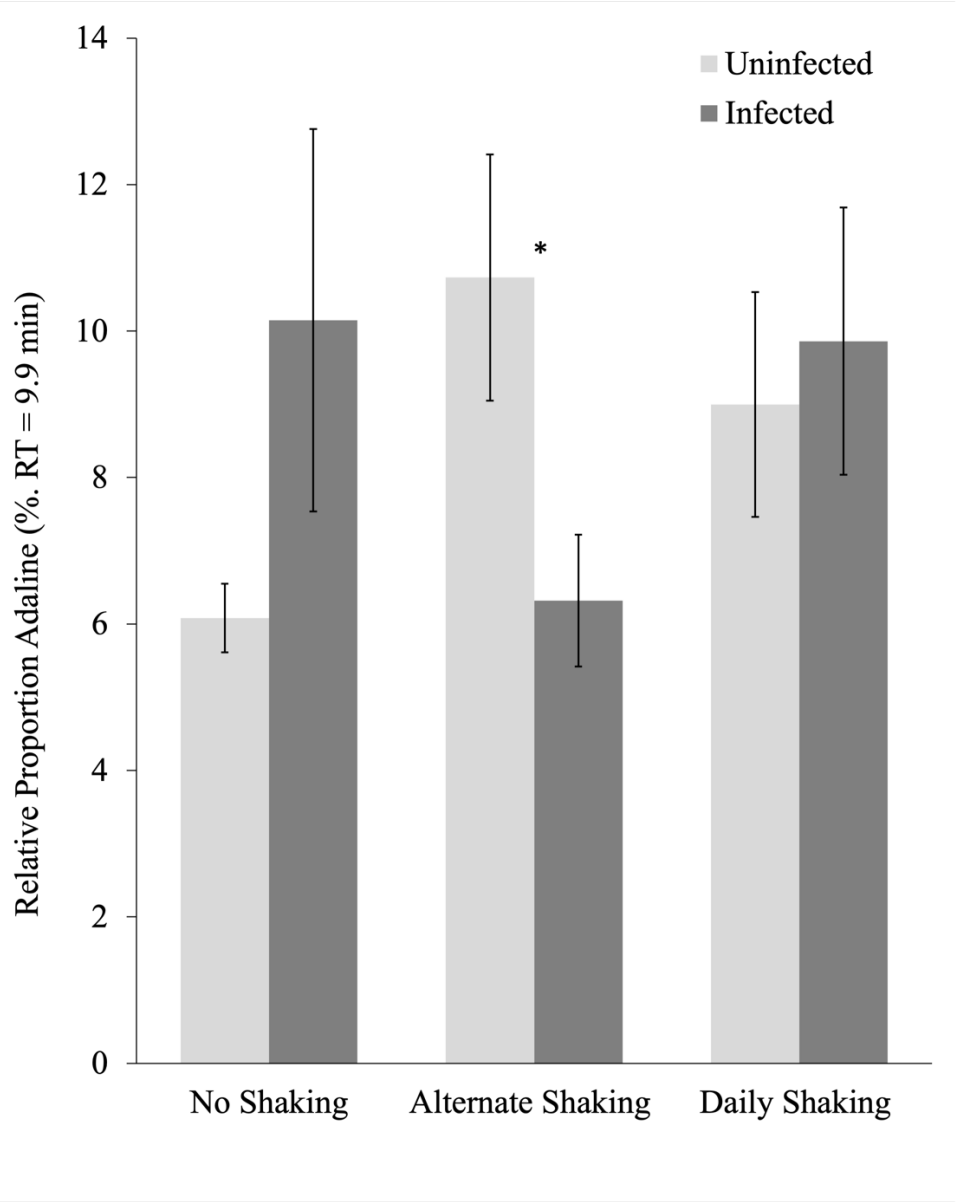


Figure 4.3. Relative percent area of adaline peak (GC-FID; RT: 9.9 min) ± standard error (SE) in hemolymph collected from *Vairimorpha adaliae*-infected *Adalia bipunctata* adults exposed to varying levels of physical agitation. Means with an asterisk (*) represent a significant difference between uninfected and infected: Control ($F = 2.59$, $df = 38$, $p = 0.568$); Alternate shaking ($F = 6.54$, $df = 38$, $p = 0.026$); Daily shaking ($F = 0.45$, $df = 38$, $p = 0.719$).

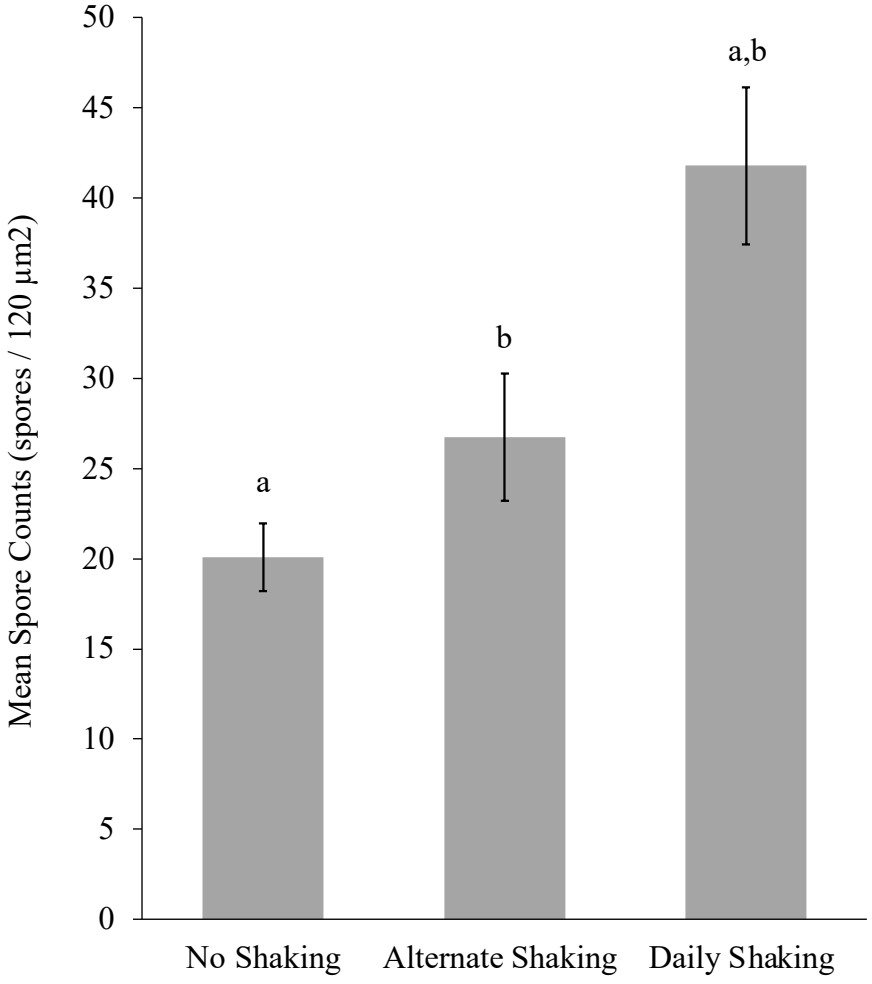


Figure 4.4. Mean spore counts (number of spores per 120 μm^2) ± standard error (SE) from *Vairimorpha*-infected *Adalia bipunctata* adults exposed to physical agitation. Means with the same letter represent a significant difference (Control and Alternate shaking: $Z = 16.86$, $p = 0.304$; Control and Daily shaking: $Z = 16.86$, $p < 0.001$; Alternate shaking and Daily shaking ($Z = 16.86$, $p = 0.003$).

5. CHAPTER FIVE

Conclusion

The aim of this study was to investigate factors that influence the development of microsporidian infections in beneficial lady beetles, as delayed larval development is the only reported consequence, thus far, for these obligate parasites found in *A. bipunctata* and *H. convergens*. Recreating laboratory conditions that are more reflective of the challenges beetles would face in nature was one part of this study. The other factor of interest was alkaloid secondary metabolites, which are used by coccinellids for anti-predation defences, and their potential role in fighting infections such as microsporidiosis. The overwhelming majority of known secondary metabolites, including alkaloids, are of plant origin, and our understanding surrounding their purpose is mostly limited to their role as anti-predation compounds. However, many plants produce antimicrobial secondary metabolites called phytoalexins that can inhibit the germination and/or growth of bacteria and fungi, as well as other plant cells (Harborne, 2002; Pedras & Yaya, 2015). For example, both wild and cultivated crucifers produce phytoalexins comprised of indole alkaloids that exhibit antimicrobial activity against plant pathogens and display anticarcinogenic properties for certain human cell lines (see Pedras et al., 2011). Secondary metabolites produced by some animals (like terpenes) share common biosynthetic pathways with those produced by plants, and some animals, such as coccinellids, appear to mimic plant defences by producing their own alkaloids (Beran et al., 2019; Harborne, 2002). Furthermore, chemically identical secondary metabolites, such as benzoquinones, monoterpenes and cyanogenic glycosides, are often found in both plants and insects, including the sesquiterpene alkene (E)-b-caryophyllene that is present in corn, lima beans, cannabis and the coccinellid *H. axyridis* (see Beran et al., 2019). Alkaloids present in lady beetles may act as the animal equivalent of phytoalexins and play a larger role in the coccinellid immune system than previously thought.

In Chapter Two, uninfected and microsporidia-infected *A. bipunctata* and *H. convergens* were provided food either daily or on alternate days to simulate the unpredictable nature of food availability these beetles would experience outside of a laboratory setting. For both species, development was delayed for individuals infected with microsporidia, as well as for those fed irregularly. *V. adaliae*-infected *A. bipunctata* larvae provided an irregular food supply underwent further developmental delays compared to their generously fed counterparts. This was not

observed for *T. hippodamiae*-infected *H. convergens*. Because inadequate nutrition has been shown to increase the susceptibility of some coccinellids to disease (James & Lighthart, 1992), one would expect limited food availability to amplify the negative effects of microsporidiosis in lady beetles. However, this was only the case for *A. bipunctata* infected with *V. adaliae*. Alkaloid content (RP adaline) was higher for *A. bipunctata* fed daily compared to those that were fed irregularly. A reduced food supply may restrain adaline production, a metabolic process, because insect metabolism is reliant on adequate nutrition (see House, 1961). RP adaline was also higher for *V. adaliae*-infected beetles, compared to uninfected, as were spore counts for infected *A. bipunctata* fed irregularly compared to those fed daily. These results suggest that adaline content is influenced by infection and that irregular food supply increases pathogen load. With respect to spore counts for *T. hippodamiae*-infected *H. convergens*, no significant difference was observed between beetles fed daily or irregularly. Unfortunately, alkaloid samples (hippodamine and convergine) collected from *H. convergens* reflex-fluid had degraded over time and could not be analyzed for alkaloid comparison. And the analysis of these alkaloids could not be repeated because access to the required analytical equipment (liquid chromatography–mass spectrometry) was no longer available. Therefore, future repetition of these trials with the inclusion of alkaloid analysis will be necessary in order to make any definite conclusions regarding the relationship between the alkaloids hippodamine and convergine, food availability and microsporidiosis in *H. convergens*.

In Chapter Three, uninfected and *V. adaliae*-infected *A. bipunctata* were reared at temperatures elevated above optimal rearing temperatures (25 °C). Lady beetles often experience a wide range of environmental temperatures in the natural environment and little is known regarding the effects of microsporidia on coccinellids when they are reared at higher or lower temperatures. As in Chapter Two, microsporidia-infected beetles took longer to develop than did uninfected beetles. However, infected larvae took longer to develop at 25 °C than at 27.5 °C or 30 °C. Alkaloid content (RP adaline) increased when larvae were reared at 30 °C. This may be a result of increased metabolic activity at temperatures elevated above 25 °C, as temperature and metabolism are correlated in insects (Denlinger & Yocum, 1998). But unlike the results reported in Chapter Two, RP adaline was not affected by *V. adaliae*. This may be a result of the high individual variation documented in *A. bipunctata* (de Jong et al., 1991) that is reflected by the standard errors observed in this study, or a result of the small sample sizes used in the current

study. Both percent infection and average spore count decreased at 27.5 °C and 30 °C. These results suggest that temperatures above 25 °C have a mitigating effect against *V. adaliae* in *A. bipunctata*. However, this study only examined a subset of constant high temperatures and future studies should consider lower and/or fluctuating temperatures to gain a broader perspective that is more reflective of the natural environment and behaviours of *A. bipunctata*, including dispersal from unfavourable conditions.

In the final chapter, Chapter Four, alkaloid content (RP adaline) was analyzed throughout *A. bipunctata* development, because the quantity of alkaloids at each life stage and the relationship between adaline and *V. adaliae* had yet to be examined. Additionally, adults were exposed to physical agitation (to represent physical stress that they may experience in nature) to determine the combined effects of physical stress and infection on adult beetles with respect to alkaloid content and severity of infection. Overall, RP adaline increased in uninfected and infected *A. bipunctata* from egg to adult. Uninfected individuals had higher RP adaline than did infected individuals during early development; however, adaline content was higher in infected *A. bipunctata* from the third-instar onwards, when compared to their uninfected counterparts. Because infected eggs had lower adaline content than uninfected eggs, one might wonder if these infected eggs are more palatable for consumption by larval cohorts or other lady beetle species, which may increase the likelihood of pathogen transmission. Following exposure to physical agitation on alternate days, adaline content was higher for uninfected adults than infected adults. The unpredictability of being shaken on alternate days may be more stressful and challenging for individuals to overcome than predictable daily shaking. Adults exposed to daily shaking had higher spore counts compared to individuals from the control and alternate shaking groups; however, these differences in pathogen load were not correlated with changes in alkaloid production. This suggests that infected beetles are unable to simultaneously combat the pathogen and produce adaline while undergoing physical stress. From a biological perspective, one would expect to observe differences in alkaloid production through coccinellid development, as each successive life stage faces different external pressures and risks. When infected with the microsporidium *V. adaliae*, however, adaline production was reduced during early development but increased significantly in late life stages.

Results of this study indicate a relationship between adaline and *V. adaliae* in *A. bipunctata*. Adaline content increased when pathogen load decreased (and vice versa), which

suggests that this alkaloid, in addition to providing protection from predation, may also influence chronic infections, such as those caused by microsporidia. When lady beetles are infected with a microsporidian pathogen, one might speculate that beetles may not allocate limited resources away from combating the pathogen to be used towards the production of anti-predation compounds (alkaloids) unless these compounds provided some antimicrobial protection. However, additional studies are needed to determine if other factors are involved (such as disruptions to metabolic functions caused by heavy infections of the insect fat body). It is also important to note that the relationship between lady beetle alkaloids and chronic infections (microsporidiosis) has been examined in only two coccinellids, *A. bipunctata* and *H. convergens* and that these studies focussed on only two pathogens that infect these hosts (*V. adaliae* and *T. hippodamiae*, respectively). Only adaline, one of the two primary alkaloids in *A. bipunctata* was examined because adalinine was not detectable with the methodology used. The two primary alkaloids, hippodamine and convergine, in *H. convergens* have yet to be evaluated.

It is important to note that relative proportions were used in this study to compare general trends in alkaloid content, as opposed to the more analytical absolute quantification. This was due to the preliminary nature of the study and available techniques/equipment, as the amount of reflex-fluid produced by individuals varied and wasn't quantified. Studies such as those by Oudendijk and Sloggett (2022) examine absolute alkaloid quantification in *A. bipunctata* (with respect to diet quality and reflex-bleeding) using precise collection, extraction, and analytical techniques. Samples were collected and extracted from whole lady beetles and eggs that were weighed and included the addition of an internal standard (a nicotine solution). Alkaloids were quantified by comparing the internal standard peak to alkaloid peaks and results were reported as milligram per mass. These techniques would be crucial in confirming the relationship between alkaloids and microsporidia in coccinellids. Additionally, because relative proportions were compared, it is possible that adaline content is staying the same across treatments and the other components of reflex-fluid are changing relative to adaline. Also worth mentioning is that this study examined relative proportions in reflex-fluid and not body hemolymph. Reflex-fluid was analyzed for ease of extraction, as well as means of specimen preservation for other aspects of the current study (adult morphometrics and pathogen spore load). However, since microsporidia are intracellular, found within the coccinellid body, the analysis of body hemolymph (as opposed to expressed reflex-fluid) would be more reflective of what these pathogens would naturally

encounter. An unfortunate limitation of the results from this study is that degraded adaline was not included in analysis. This was an unintentional oversight, as the inclusion of degraded peaks may influence the results observed in this study and could provide further insight into the relationship between alkaloids and microsporidia in *A. bipunctata*. Future studies should ensure that all degraded alkaloid peaks are included in alkaloid analyses and comparisons.

5.1 Knowledge Transfer:

To aid in the dissemination of relevant information to fellow researchers and industry, chapters of my thesis have been published and/or submitted for publication as follows:

1. Steele, T., Bjørnson, S., 2019. Effects of microsporidiosis and food availability on the two- spotted lady beetle, *Adalia bipunctata* L., and convergent lady beetle *Hippodamia convergens* Guérin-Méneville. Journal of Invertebrate Pathology 161, 7–13.
2. Steele, T., Singer, R. D., Bjørnson, S. 2020. Effects of food availability on microsporidiosis and alkaloid production in the two-spotted lady beetle, *Adalia bipunctata* L. Journal of Invertebrate Pathology 175, 107443.
3. Steele, T., Singer, R. D., Bjørnson, S. 2020. Effects of temperature on larval development, alkaloid production and microsporidiosis in the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae). Journal of Invertebrate Pathology 172, 107353.
4. Steele, T., Singer, R. D., Bjørnson, S. 2023. Alkaloid content in microsporidia-infected *Adalia bipunctata* (Coleoptera: Coccinellidae) life stages, and pathogen spore load in adults after exposure to physical stress. Journal of Invertebrate Pathology (Submitted January 2023).

The information gained from this project may be useful to those who mass-rear and/or collect beneficial lady beetles for biological pest control and could provide insights regarding the health and quality of these natural enemies with respect to the role alkaloids may naturally play in keeping microbial infections low and the impact of rearing conditions on the development of disease, such as microsporidiosis. This research adds to our knowledge regarding some of the factors that may cause the inconsistent and unpredictable effects of microsporidia on their hosts.

Because many questions remain regarding microsporidia and the relationship between these pathogens and the potential protective properties of coccinellid alkaloids, this preliminary

exploratory research provides fundamental information for the development of future research. Before this project was undertaken, adaline in two-spotted lady beetles was known only for its role in anti-predation defences. The data herein shows an association between infection and adaline content, which suggests that coccinellid alkaloids, such as adaline in *A. bipunctata*, may help protect against chronic microsporidiosis and is impacted by various abiotic stressors. Future research should be undertaken to confirm the relationship between coccinellid alkaloids and microsporidiosis more closely and to confirm the antimicrobial activity of alkaloids in coccinellids. This could include:

1. Revisiting the alkaloids of interest in the current study (including the minor alkaloid adalinine in *A. bipunctata* and both major and minor alkaloids, convergine and hippodamine, in *H. convergens*) that were not detectable with the methodology used or with equipment available at the time. Information gained would help complete our understanding surrounding the role of these alkaloids as antimicrobial defences against pathogens such as microsporidia in *A. bipunctata* and *H. convergens*.
2. Analytical quantifications of coccinellid alkaloids in uninfected vs. microsporidia-infected beetles using internal standards and high-tech analytical GC equipment (not available at Saint Mary's University at the time of this project). The current study was limited to the comparison of relative proportions of alkaloids, but the information gained from future work with different analytical equipment would determine precise quantities of alkaloids in lady beetle developmental stages, and during various trials.
3. Comparing alkaloid content in lady beetles infected with a known and/or varying dose of microsporidian spores. One limitation of the current study is that the doses of microsporidia were unknown. Infections were initiated through the consumption of a single infected egg, which is what would happen in nature. However, studies that are based on infection that is caused by a known spore dose would be valuable to determine how alkaloid content is influenced by level of infection.
4. Evaluating the effects of other stress factors, such as crowding and cold temperatures, on the development of microsporidiosis and production of alkaloids in lady beetles to provide further insight on the inconsistencies of these pathogens and the factors that may influence the severity of infection.

5. Studying the effects of alkaloid production and microsporidiosis on other coccinellid species. Because the current study examined adaline in *A. bipunctata* (a beneficial species), examining alkaloid production from invasive species (such as *H. axyridis* and *C. septempunctata*) in combination with microsporidiosis or other chronic infections may provide information relevant to the success of these beetles at outcompeting native species.

According to the literature, lady beetles produce types of alkaloids that have yet to be identified in plants (Harborne, 2002). A detailed investigation to determine if this statement holds true would make for an interesting, in-depth project. Students with a strong background in chemistry could analyze and compare lady beetle alkaloids with plant alkaloids, and examine the chemical structure, biosynthesis, evolutionary relationship and bioactive properties of these compounds. Additionally, it would be worth investigating if other natural products (such as manuka honey, see Bjørnson et al., 2023) or secondary metabolites (of plant and/or animal origin) influence the production of alkaloids by lady beetles and the development of microsporidiosis.

Alkaloids from plants have received much attention with respect to research and human applications, with many medications having plant-alkaloid origins (see Heinrich et al., 2021). Because plant and animal alkaloids share common biosynthetic pathways and are both produced as defensive agents, alkaloids of animal origin should receive equivalent attention as do plant alkaloids with respect to research and potential applications. Alkaloids of coccinellid origin may prove useful for pest control as a means to deter insect pests or as agents that improve the overall health of commercially available natural enemies. Harmonine, the major alkaloid of *H. axyridis* has shown broad-spectrum antimicrobial activity against several human pathogens, including: a strain of drug-sensitive *Staphylococcus aureus*; a multi-resistant *S. aureus* (MRSA) strain; four strains of fast-growing mycobacteria; and the asexual blood stages of *Plasmodium falciparum*, the pathogen responsible for malaria tropica (see Röhrich et al., 2012). It would be interesting to learn if harmonine shows antimicrobial properties against other human pathogens and if the alkaloids from *A. bipunctata* exhibit similar antimicrobial traits. If a medical or commercial application for coccinellid alkaloids becomes apparent, mass-production of these compounds could follow because most known lady beetle alkaloids have been synthesized in laboratory settings.

I was fortunate and had the opportunity to attend and present my research at a joint conference for the Entomological Society of America, the Entomological Society of Canada, and the Entomological Society of British Columbia during my final year of study. The theme of this conference was *Entomology as Inspiration: Insects through art, science and culture*, and included an objective to promote female and First Nation speakers. Keynote speakers expressed the importance and value of indigenous knowledge in entomology and discussed how we can access and incorporate this unique and often overlooked wealth of information into our research. One indigenous researcher, who was studying dung beetles that are located within the ancestral First Nations territory in Colorado, shared his personal story of how he incorporated his culture into his research, and explained how the vast information held by First Nations people in the area was beneficial to his project by providing previously unknown data. He concluded by saying "words are meaningless without action; recognizing First Nations land is more than just making the statement, it's about respect". He stated that "if we're going to conduct research on another peoples' land, using their knowledge, we should try to include them as true collaborators (not just an acknowledgement)". Students and researchers may wish to consider this when designing future projects, if at all possible.

Saint Mary's University recognises that we are located on Mi'kma'ki, the ancestral and unceded territory of the Mi'kmaq people. The Mi'kmaq share their cultural views that non-human animals are persons and siblings, that reciprocity is essential in maintaining the human-animal relationship and, with respect to the environment, it is important that animals do not live in scarcity (Robinson, 2014). I am interested to know what knowledge Mi'kmaq viewpoints hold regarding lady beetles, their natural protective compounds and their antimicrobial properties (particularly those found locally in Nova Scotia), and how this knowledge could be incorporated into my research area.

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Appendix

GC-MS/FID Summary Data: Relative percent area of adaline peak (FID) for individual samples collected from uninfected and *Vairimorpha adaliae*-infected *Adalia bipunctata* used in the current study, arranged by chapter. Adaline was identified by comparison with mass-spectra from the literature and was found at a retention time of 9.9 minutes (MS).

Chapter	Treatment	Sample ID	FID % Area	Treatment	Sample ID	FID % Area
2. Food Availability	Uninfected Control	AB-QC-01	29.10	Infected Control	ABC-01	46.40
		AB-QC-03	32.40		ABC-04	44.00
		AB-QC-02	27.30		ABC-06	42.50
		AB-QC-04	22.50		ABC-07	18.50
		AB-QC-06	15.20		ABC-09	27.10
		AB-QC-07	20.10		ABC-10	23.20
		AB-QC-09	30.60		ABC-11	43.80
		AB-QC-10	39.30		ABC-12	29.90
		AB-QC-11	32.10		ABC-15	42.50
		AB-QC-2B	23.70		ABC-16	37.40
		Average =	**27.23**		**Average =**	**35.53**
	Uninfected Treatment	AB-UT-01	16.20	Infected Treatment	ABT-01	37.00
		AB-UT-03	26.10		ABT-04	21.20
		AB-UT-05	0.00		ABT-07	36.80
		AB-UT-06	16.60		ABT-08	41.40
		AB-UT-07	0.00		ABT-10	27.20
		AB-UT-09	33.00		ABT-12	35.40
		AB-UT-11	13.00		ABT-13	37.90
		AB-UT-12	0.00		ABT-19	34.60
		AB-UT-1A	19.80		ABT-20	22.50
		AB-UT-8B	12.10		ABT-23	31.10
		Average =	**13.68**		**Average =**	**32.51**
3. Temperature	Uninfected 25°C	ABU-25-01	0.00	Infected 25°C	ABI-25-02	24.40
		ABU-25-03	38.20		ABI-25-03	36.90
		ABU-25-04	39.60		ABI-25-06	0.00
		ABU-25-05	36.90		ABI-25-07	0.00
		ABU-25-07	17.30		ABI-25-10	39.80
		ABU-25-13	25.20		ABI-25-11	36.50
		ABU-25-16	30.40		ABI-25-04	50.00
		ABU-25-02	25.70		ABI-25-13	46.70
		ABU-25-06	20.40		ABI-25-14	29.90
		ABU-25-17	38.60		ABI-25-16	21.00
		Average =	**27.23**		**Average =**	**28.52**
	Uninfected 27.5°C	ABU-27-03	19.90	Infected 27.5°C	ABI-27-05	28.40
		ABU-27-04	43.70		ABI-27-04	46.10
		ABU-27-01	25.70		ABI-27-01	26.10
		ABU-27-02	39.40		ABI-27-09	47.30
		ABU-27-10	8.60		ABI-27-10	31.70
		ABU-27-12	56.80		ABI-27-17	42.40
		ABU-27-06	15.30		ABI-27-22	31.60
		ABU-27-07	46.20		ABI-27-29	42.50
		ABU-27-08	21.10		ABI-27-06	30.30
		ABU-27-05	23.60		ABI-27-08	34.50
		Average =	**30.03**		**Average =**	**36.09**

Chapter	Treatment	Sample ID	FID % Area	Treatment	Sample ID	FID % Area
	Uninfected 30°C	ABU-30-04	29.80	Infected 30°C	ABI-30-13	43.90
		ABU-30-06	24.60		ABI-30-14	63.00
		ABU-30-08	35.80		ABI-30-19	34.70
		ABU-30-02	53.70		ABI-30-01	30.20
		ABU-30-03	46.30		ABI-30-02	60.80
		ABU-30-09	61.70		ABI-30-04	33.90
		ABU-30-10	47.00		ABI-30-05	53.50
		ABU-30-11	62.70		ABI-30-07	22.40
		ABU-30-18	41.40		ABI-30-10	55.00
		ABU-30-22	47.90		ABI-30-12	49.70
		Average =	**45.09**		**Average =**	**44.71**
4. Development	Uninfected Egg	EGG 1	3.80	Infected Egg	EGG 1	0.00
		EGG 2	2.62		EGG 2	4.11
		EGG 3	4.02		EGG 3	1.97
		EGG 4	0.00		EGG 4	0.00
		EGG 5	0.00		EGG 5	0.00
		EGG 6	0.00		EGG 6	0.00
		EGG 7	3.10		EGG 7	0.00
		EGG 8	0.00		EGG 8	1.94
		EGG 9	4.68		EGG 9	0.00
		EGG 10	0.00		EGG 10	0.00
		EGG 11	2.83		EGG 11	3.58
		EGG 12	2.77		EGG 12	2.08
		EGG 13	3.54		EGG 13	0.00
		EGG 14	3.21		EGG 14	0.00
		EGG 15	4.65		EGG 15	0.00
		EGG 16	4.68		EGG 16	0.00
		EGG 17	3.52		EGG 17	0.00
		EGG 18	4.44		EGG 18	0.00
		EGG 19	1.75		EGG 19	0.00
		EGG 20	3.23		EGG 20	0.00
		Average =	**2.64**		**Average =**	**0.68**
	Uninfected 1st Instar	L1-1	0.00	Infected 1st instar	L1-1	20.81
		L1-2	0.00		L1-2	8.52
		L1-3	0.00		L1-3	0.00
		L1-4	0.00		L1-4	0.00
		L1-5	6.86		L1-5	0.00
		L1-6	14.59		L1-6	6.20
		L1-7	9.62		L1-7	8.75
		L1-8	3.95		L1-8	0.00
		L1-9	27.14		L1-9	0.00
		L1-10	4.00		L1-10	0.00
		L1-11	3.82		L1-11	10.41
		L1-12	2.00		L1-12	4.23
		L1-13	3.71		L1-13	10.63
		L1-14	5.99		L1-14	0.00
		L1-15	3.89		L1-15	0.00
		L1-16	8.35		L1-16	1.68
		L1-17	5.18		L1-17	0.00
		L1-18	9.03		L1-18	0.00
		L1-19	3.38		L1-19	0.00
		L1-20	3.77		L1-20	0.00
		Average =	**5.76**		**Average =**	**3.56**

Chapter	Treatment	Sample ID	FID % Area	Treatment	Sample ID	FID % Area
	Uninfected 2nd Instar	L2-1	0.00	Infected 2nd Instar	L2-1	0.00
		L2-2	22.00		L2-2	0.00
		L2-3	3.67		L2-3	0.00
		L2-4	6.20		L2-4	0.00
		L2-5	2.99		L2-5	0.00
		L2-6	4.01		L2-6	0.00
		L2-7	2.43		L2-7	39.15
		L2-8	9.56		L2-8	0.00
		L2-9	2.53		L2-9	1.04
		L2-10	1.46		L2-10	0.00
		L2-11	4.10		L2-11	4.98
		L2-12	7.43		L2-12	4.21
		L2-13	8.91		L2-13	0.00
		L2-14	4.59		L2-14	33.58
		L2-15	4.14		L2-15	0.00
		L2-16	4.01		L2-16	0.00
		L2-17	12.11		L2-17	0.00
		L2-18	14.65		L2-18	4.71
		L2-19	8.99		L2-19	8.63
		L2-20	5.14		L2-20	12.57
		Average =	**6.45**		**Average =**	**5.44**
	Uninfected 3rd Instar	L3-1	10.08	Infected 3rd Instar	L3-1	21.29
		L3-2	0.00		L3-2	2.89
		L3-3	4.00		L3-3	27.13
		L3-4	2.82		L3-4	3.29
		L3-5	4.46		L3-5	0.00
		L3-6	1.83		L3-6	12.78
		L3-7	3.43		L3-7	5.54
		L3-8	2.32		L3-8	0.00
		L3-9	2.46		L3-9	2.48
		L3-10	8.80		L3-10	1.61
		L3-11	25.66		L3-11	11.73
		L3-12	8.68		L3-12	17.88
		L3-13	12.14		L3-13	21.62
		L3-14	21.09		L3-14	5.48
		L3-15	4.85		L3-15	0.00
		L3-16	9.58		L3-16	28.25
		L3-17	2.90		L3-17	28.05
		L3-18	3.43		L3-18	34.89
		L3-19	3.12		L3-19	45.52
		L3-20	8.89		L3-20	19.59
		Average =	**7.03**		**Average =**	**14.50**
	Uninfected 4th Instar	L4-1	6.80	Infected 4th Instar	L4-1	3.02
		L4-2	2.31		L4-2	41.32
		L4-3	13.69		L4-3	16.57
		L4-4	6.08		L4-4	4.75
		L4-5	28.77		L4-5	15.01
		L4-6	4.45		L4-6	1.62
		L4-7	1.57		L4-7	48.42
		L4-8	15.22		L4-8	2.46
		L4-9	3.21		L4-9	8.39
		L4-10	4.78		L4-10	38.18
		L4-11	14.88		L4-11	8.31
		L4-12	14.87		L4-12	8.31

Chapter	Treatment	Sample ID	FID % Area	Treatment	Sample ID	FID % Area
		L4-13	8.36		L4-13	45.55
		L4-14	13. 068		L4-14	57.71
		L4-15	0.00		L4-15	40.55
		L4-16	6.04		L4-16	20.38
		L4-17	12.70		L4-17	0.00
		L4-18	12.27		L4-18	3.78
		L4-19	10.16		L4-19	55.40
		L4-20	30.58		L4-20	43.06
		Average =	**10.35**		**Average =**	**23.14**
	Uninfected Pupa	PUPA 1	22.17	Infected Pupa	PUPA 1	25.48
		PUPA 2	5.68		PUPA 2	20.30
		PUPA 3	12.99		PUPA 3	28.12
		PUPA 4	19.73		PUPA 4	28.15
		PUPA 5	3.00		PUPA 5	23.17
		PUPA 6	19.87		PUPA 6	13.80
		PUPA 7	13.36		PUPA 7	12.91
		PUPA 8	14.09		PUPA 8	14.69
		PUPA 9	12.33		PUPA 9	10.78
		PUPA 10	12.72		PUPA 10	3.14
		PUPA 11	37.58		PUPA 11	16.72
		PUPA 12	21.63		PUPA 12	19.90
		PUPA 13	7.04		PUPA 13	23.83
		PUPA 14	6.27		PUPA 14	55.24
		PUPA 15	25.88		PUPA 15	25.47
		PUPA 16	48.35		PUPA 16	37.18
		PUPA 17	10.12		PUPA 17	49.65
		PUPA 18	15.86		PUPA 18	33.13
		PUPA 19	4.47		PUPA 19	23.02
		PUPA 20	6.99		PUPA 20	28.14
		Average =	**16.01**		**Average =**	**24.64**
	Uninfected Adults	ADULT 1	18.63	Infected Adults	ADULT 1	16.72
		ADULT 2	23.85		ADULT 2	62.66
		ADULT 3	5.36		ADULT 3	3.91
		ADULT 4	21.65		ADULT 4	21.31
		ADULT 5	4.12		ADULT 5	28.82
		ADULT 6	4.87		ADULT 6	18.84
		ADULT 7	13.29		ADULT 7	10.05
		ADULT 8	7.44		ADULT 8	39.91
		ADULT 9	16.00		ADULT 9	14.96
		ADULT 10	13.91		ADULT 10	23.29
		ADULT 11	11.91		ADULT 11	36.41
		ADULT 12	19.03		ADULT 12	14.55
		ADULT 13	18.92		ADULT 13	36.41
		ADULT 14	5.71		ADULT 14	14.55
		ADULT 15	5.28		ADULT 15	30.94
		ADULT 16	7.65		ADULT 16	28.47
		ADULT 17	5.93		ADULT 17	29.25
		ADULT 18	13.09		ADULT 18	25.34
		ADULT 19	11.54		ADULT 19	37.78
		ADULT 20	16.11		ADULT 20	51.02
		Average =	**12.21**		**Average =**	27.26
4. Agitation	Uninfected Control	ABU-16	5.11	Infected Control	ABI-24	5.03
		ABU-13	3.12		ABI-25	2.57

Chapter	Treatment	Sample ID	FID % Area	Treatment	Sample ID	FID % Area
		ABU-42	3.58		ABI-43	4.07
		ABU-64	4.51		ABI-21	3.20
		ABU-89	48.34		ABI-67	3.27
		ABU-15	5.83		ABI-16	3.01
		ABU-41	8.69		ABI-13	3.82
		ABU-98	5.88		ABI-63	3.91
		ABU-114	6.21		ABI-38	3.07
		ABU-93	5.38		ABI-72	4.44
		ABU-25	9.32		ABI-23	4.88
		ABU-50	2.92		ABI-42	6.59
		ABU-75	10.41		ABI-18	31.13
		ABU-38	6.66		ABI-65	4.45
		ABU-45	6.20		ABI-70	45.64
		ABU-22	3.96		ABI-17	19.41
		ABU-71	7.62		ABI-41	8.58
		ABU-20	5.68		ABI-63	26.64
		ABU-74	7.11		ABI-100	5.87
		ABU-41	7.31		ABI-122	13.33
		Average =	**6.08**		**Average =**	**10.15**
	Uninfected Alternate	ABU-49	9.30	Infected Alternate	ABI-18	1.84
		ABU-46	17.04		ABI-48	5.26
		ABU-65	3.00		ABI-41	6.32
		ABU-47	5.49		ABI-39	4.54
		ABU-97	5.88		ABI-69	3.76
		ABU-24	25.74		ABI-17	3.76
		ABU-50	5.15		ABI-40	3.97
		ABU-68	4.59		ABI-49	10.13
		ABU-73	20.06		ABI-64	19.63
		ABU-88	3.99		ABI-11	5.39
		ABU-46	9.48		ABI-16	7.05
		ABU-18	17.66		ABI-13	2.44
		ABU-40	9.98		ABI-68	8.55
		ABU-67	2.80		ABI-74	4.00
		ABU-66	8.98		ABI-66	6.24
		ABU-19	28.87		ABI-43	5.67
		ABU-49	4.43		ABI-15	4.37
		ABU-63	10.66		ABI-64	5.35
		ABU-48	12.91		ABI-92	12.75
		ABU-97	8.62		ABI-125	5.34
		Average =	**10.73**		**Average =**	**6.32**
	Uninfected Daily	ABU-25	4.92	Infected Daily	ABI-23	3.49
		ABU-67	4.65		ABI-47	2.75
		ABU-22	7.28		ABI-22	5.10
		ABU-44	12.33		ABI-90	10.28
		ABU-90	4.56		ABI-45	3.21
		ABU-17	3.66		ABI-15	3.31
		ABU-43	5.92		ABI-19	5.53
		ABU-40	18.06		ABI-74	27.50
		ABU-39	7.71		ABI-50	30.97
		ABU-99	6.68		ABI-113	4.77
		ABU-64	3.63		ABI-89	11.56
		ABU-65	12.51		ABI-46	5.96
		ABU-42	12.46		ABI-38	9.21
		ABU-44	5.33		ABI-91	14.25

Chapter	Treatment	Sample ID	FID % Area	Treatment	Sample ID	FID % Area
		ABU-88M	11.83		ABI-50M	4.92
		ABU-47F	30.76		ABI-14F	21.67
		ABU-17F	14.35		ABI-49F	4.62
		ABU-68F	10.60		ABI-96F	13.99
		ABU-43F	0.00		ABI-24F	6.41
		ABU-72	2.67		ABI-39F	7.70
		Average =	**9.00**		**Average =**	**9.86**

Example of Unedited GC-FID Output: Percent area of all peaks (FID) in *Adalia bipunctata* hemolymph from uninfected and *Vairimorpha adaliae*-infected individuals reared at 25 °C. Adaline was identified by comparison with mass-spectra from the literature and was found at a retention time of 9.9 minutes (MS).

Sample: ABU-25-03 GC-FID: Output

Retention Time (min.)	Area	Signal/Noise	Scan Description
4.2	20083	289	Merged, RIC
4.5	4685	276	Merged, RIC
5.3	2823	194	Merged, RIC
6.0	2937	297	Merged, RIC
6.6	5415	315	Merged, RIC
6.7	4261	127	Merged, RIC
7.1	5435	61	Merged, RIC
7.4	26123	387	Merged, RIC
8.7	22816	708	Merged, RIC
9.9	**62584**	**1364**	**Merged, RIC**
10.7	39530	1005	Merged, RIC

Sample: ABI-25-03 GC-FID: Output

Retention Time (min.)	Area	Signal/Noise	Scan Description
4.5	3149	154	Merged, RIC
6.0	2758	125	Merged, RIC
6.6	4935	179	Merged, RIC
6.7	3655	155	Merged, RIC
7.4	22296	411	Merged, RIC
8.7	19598	434	Merged, RIC
9.9	**61331**	**929**	**Merged, RIC**
10.8	38285	903	Merged, RIC
12.7	3892	277	Merged, RIC
14.6	23852	0	Merged, RIC
14.8	10560	1	Merged, RIC