Effects of sweet fennel, *Foeniculum vulgare* var. *dulce*, essential oil on the microsporidian pathogen *Vairimorpha adaliae* and its host, the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae)

by

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Abstract

The two-spotted lady beetle, *Adalia bipunctata* L., is commercially available for biological pest control in Europe and North America. It is susceptible to the microsporidium *Vairimorpha adaliae*, a spore-forming fungal pathogen which prolongs larval development. Sweet fennel essential oil, *Foeniculum vulgare* var. *dulce*, is known to have antifungal properties and has been used for the management of both the microsporidium *Nosema ceranae* in honeybees and the late blight pathogen, *Phytophthora infestans*, in tomato and potato plants. However, sweet fennel essential oil has not been investigated for the management of *V. adaliae* in *A. bipunctata*. In this study, the effects of sweet fennel essential oil on larval development time, larval mortality rate, adult sex ratio, and microsporidian infection (spore counts) in *V. adaliae*-infected *A. bipunctata* were examined. Development time, mortality rate, and sex ratio of larvae fed green peach aphids, *Myzus persicae*, and water did not differ significantly from larvae fed aphids and 10%, 20%, or 30% fennel, respectively. Although spore loads did not differ significantly, heavier infections were observed in increased fennel concentrations with an increase of 12.83%, 61.33%, and 58.24% in the 10%, 20%, and 30%, respectively. This study suggests that fennel essential oil is safe to use on *A. bipunctata* and may provide additional energy to the host beetle to withstand the effects of the microsporidian pathogen as larval development time was unaffected, despite heavier infections. Understanding the role of plant secondary compounds on pathogens in lady beetles will help to ensure that beneficial insects remain effective biological control predators as an alternative to synthesized pesticides.

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1. Introduction

1.1 Biological Control

Natural control involves the maintenance of insect populations at densities below a level that causes economic damage to crops and livestock. This often occurs in nature, and the process is the result of a combination of an abiotic factor including environmental conditions, and a biotic factor including natural enemies (DeBach and Rosen, 1991). Natural enemies consist of parasitoids, predators, and pathogens that decrease the reproductive potential or kill destructive insect pests, to reduce their population below the level that causes economic damage. Parasitoids are insects that live and feed in or on other invertebrate hosts, whereas predators tend to attack, kill, and feed on prey. Pathogens are microorganisms that cause disease by impairing the normal activities of host tissues or cells (Flint et al., 1998). The study and use of natural enemies for regulating pest population densities was introduced as the biological control method by Smith (1919). The biological control method is a form of natural pest control used in agroecosystems such as farms, greenhouses, and orchards. It consists of three main types of biological control: classical, conservation, and augmentative (Perdikis et al., 2008).

Classical biological control is an effective approach for controlling pests that have been introduced from another country or region into a new land where natural controls do not yet exist. The process involves the identification of the country of origin of the pest, foreign exploration to this region to identify the natural enemies that control the pest population in that region, and finally, the importation and introduction of exotic natural enemies to the new region to reduce the pest population to a density that no longer causes economic damage (Dixon, 2000). The first successful case of classical biological control was achieved in 1889, when the vedalia beetle, *Rhodolia cardinalis* Mulsant, was imported from Australia and subsequently introduced in

California to regulate the cottony cushion scale, *Icerya purchasi* Maskell, on California citrus (Caltagirone and Doutt, 1989). At the time, *I. purchasi* was a devastating pest of the Californian citrus industry, which had held great promise as a major economic source in California. In 1887, Charles Valentine Riley, an Entomologist for the United States Department of Agriculture and Chief of the Division of Entomology, addressed the Fruit Grower's Convention to suggest that the cottony cushion scale problem could be alleviated by sending someone to Australia to seek out and import its natural enemies into California. Riley selected his assistant Albert Koebele to travel and collect specimens from Australia as well as Daniel Coquillet to receive and culture the collected insects in Los Angeles (Caltagirone and Doutt, 1989).

In 1888, Koebele sailed to Australia and later found *R. cardinalis* feeding on *I. purchasi* (Caltagirone and Doutt, 1989). At once, he sent a total of 129 *R. cardinalis* adult specimens to Coquillet in Los Angeles, which were received and placed under a tent on an *I. purchasi*-infested orange tree. Inside the tent, the beetles were reared undisturbed and after a few months, one side of the tent was removed to allow the beetles to spread onto nearby trees (Essig, 1931; Caltagirone and Doutt, 1989). At the same time, Coquillet began sending out *R. cardinalis* colonies directly to the infested orchards in other areas of California. By mid-1889, Coquillet had distributed 10,555 specimens to 228 different orchardists. Within a year, the shipments of oranges from Los Angeles County increased from 700 to 2000 railroad carloads and by 1892 the orchards that had been rendered comparatively worthless were restored to their former condition, practically free of *I. purchasi* as a result (Caltagirone and Doutt, 1989; Essig, 1931).

The astounding success of *R. cardinalis* in California resulted in a newfound interest in biological control and was soon replicated with colonies of lady beetles introduced to control other economically damaging pests elsewhere. From 1891 to 1892, Koebele sent to California

from Australia, New Zealand, New Caledonia, and Fiji, approximately 40,000 specimens of lady beetles consisting of 40 species to control scale and mealybugs on citrus (Caltagirone and Doutt, 1989). These beetles from California were colonized directly or through other areas including Egypt, Portugal, Puerto Rico, the Philippines, and Argentina. Their successful colonization of places with differing climates from those of California or Australia suggests the enormous flexibility of lady beetles to live in varying environments (Caltagirone and Doutt, 1989). Due to the overwhelming success of *R. cardinalis* over many years, several other lady beetle species are widely used for biological control. For example, the translocation of the seven-spotted lady beetle, *Coccinella septempunctata* L., from wheat to cotton crops for control of cotton aphid, *Aphis gossypii* Glover, in China (Dixon, 2000) and the release of field-collected two-spotted lady beetles, *Adalia bipunctata* L. and *C. septempunctata* for control of aphid infestations in orchards in southeastern Kazakhstan (Hodek, 1973).

Conservation biological control is another means of biological control whereby existing natural enemy populations are maintained by conserving their local environment in instances where it is being threatened, such as through human disturbances. This approach is effective in areas where specific natural enemies already exist, but some care is required to ensure that they are maintained at levels that are high enough for the control of local pests. Conservational practices may include avoiding harmful agricultural practices such as slash-and-burn agriculture or the use of synthetic pesticides (Hodek, 1973). Conservation biological control may solely consist of this first phase, or it may incorporate augmentative biological control (the release of additional natural enemies) to increase natural enemy populations in more favourable environments (Hodek, 1973).

Augmentative biological control involves the release of additional natural enemies that are already present in a given region when too few are present to control a pest effectively. This method involves the introduction of specific, mass-released natural enemies into a known area where a particular pest is present. The population of the natural enemy is artificially increased, allowing for continual domination and attack of the targeted hosts to manage their population below the level of economic damage (Smith, 1919). Similarly, inundative control is a specific case of augmentative biological control that involves the introduction of mass-reared or field-collected natural enemies into controlled or isolated environments, such as greenhouses. Inundative control results in an immediate reduction or extermination of the pest population within the isolated environment (Hodek and Honěk, 1996). For example, the mass-rearing and release of the lady beetle, *Cryptolaemus montrouzieri* Mulsant, has been effective at controlling outbreaks of the mealybug, *Pseudococcus citri* Risso, on both citrus in California and flower crops in greenhouses (Dixon, 2000; Hodek and Honěk, 1996).

1.2 Beneficial Lady Beetles

Lady beetles are beneficial insects which prey on plant pests with approximately 179 different species intentionally imported and released in North America for biological control (Gordon, 1985). Lady beetles are referred to historically as “Coccinellids Aphidiphages” meaning they prefer to feed on a wide range of aphid species. However, when aphids are scarce or absent, lady beetles will feed on other insects, such as other Coleoptera, including coccids and diapsids (scale insects), and the immature stages of Lepidoptera and Hymenoptera (Gordon, 1985; Steele and Bjornson, 2014). For example, beetles from the genera *Coleomegilla* can survive on a variety of foods, as well as complete their development on an unusual diet of mites (Gordon, 1985).

Lady beetles are effective biological control predators because they occupy the same habitats and niches of their prey (Hodek, 1973). *A. bipunctata* larvae are positively phototactic (are attracted towards light), which enhances a positive visual orientation toward aphid colonies on host plants, which are also positively phototactic (Omkar and Pervez, 2005). This allows them to feed on aphids at the beginning of an infestation before other aphidophagous predators which use stimulus from the smell of aphid colonies to locate prey (Hodek, 1973).

In addition, lady beetle larvae can survive periods of starvation by adjusting their rate of development and adult weight to the availability of food. For example, *C. septempunctata* larvae can complete larval development on a food consumption artificially reduced by 55% to 45% by limiting their daily supply of food in the 4th larval instar to 30 or even 10 black bean aphids, *Aphis fabae* Scopoli (Hodek, 1973). Additionally, similar observations have been made for *C. septempunctata* fed *Myzus persicae* Sulzer where the food consumption was reduced to one-third of the normal amount and most larvae were still able to pupate, but the emergence of adults was low, with emerged adults smaller and producing fewer eggs (Sundby, 1966). The ability of both larvae and adult lady beetles to feed on aphids is important for effective biological control as low aphid consumption will reduce adult emergence, and in turn hinder their ability for pest attack on a host plant (Hodek, 1973).

Another advantage of lady beetles is their ability to survive starvation in the absence of aphids by consuming alternative foods including coccids, diapsids, pollen, and nectar (Omkar and Pervez, 2005). However, while alternative foods are a source of energy and increase survival, essential consumption of aphids enables larval development with lower mortality and higher oviposition rates (Hodek and Honěk, 1996).

1.3 Two-Spotted Lady Beetle: Adalia bipunctata L.

The two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae) is mainly an aphidophagous lady beetle native to Europe, Central Asia, and North America. In European populations, *A. bipunctata* adults exhibit black (melanic) elytra (hindwings) with one red spot per elytron; however, in North America, the same species exhibits light red (non-melanic) elytra, with one black spot per elytron (Omkar and Pervez, 2005; Hodek, 1973). Melanic forms exist in other *Adalia* species and are most common in *A. quadrimaculata* and *A. sexpustulata* which have black elytra with four or six red spots, respectively. The most common non-melanic forms are *Adalia typica* and *A. annulate* which have red elytra with two black spots or irregular patches, respectively (Omkar and Pervez, 2005).

A. bipunctata feed on several aphid species including the green peach aphid, *M. persicae* (Omkar and Pervez, 2005). Neonate *A. bipunctata* larvae are attracted to host plants following the release of chemical compounds from *M. persicae* after they have been fed upon by conspecific *A. bipunctata* larvae. These compounds consist largely of the aphid alarm pheromone, *β*-farnesene, which continues to attract neonates allowing them to share aphids with other *A. bipunctata* larvae (Omkar and Pervez, 2005). The practice of social feeding increases the probability that all larvae will survive to the next instar by reducing the variation in food consumption of each individual larva. In addition to feeding on aphids, neonates commonly partake in sibling cannibalism as the larvae remain clustered on the clutch of eggs for up to one day following hatching (Omkar and Pervez, 2005).

Female larvae of *A. bipunctata* consume more aphids than male larvae. Although they have similar developmental rates, *A. bipunctata* males are smaller than females. Smaller males

spend less time feeding and more time mating when compared to larger males (Omkar and Pervez, 2005).

Female *A. bipunctata* use behavioural cues, predominantly movement to attract males (such as stopping as soon as they are touched and slightly lifting the tip of their abdomen in response to a nearby male) and mate several times to fertilize all their eggs (Hemptinne et al., 1998; Omkar and Pervez, 2005). Eggs are laid in clusters and after they hatch, the larvae develop through four larval instars into a pupa. Elytra colouration changes as the beetle becomes sclerotized (hardens). Elytra of newly emerged non-melanic adults are pale yellow and lack a distinct pattern. Gradually, the red colour of the elytra develops and darkens, and spots become apparent, giving beetles their characteristic appearance over several hours (Hodek, 1973). The red and black colouration is an example of aposematic (warning) colouration, which is accompanied by a chemical defence (production of alkaloids) to deter microbes and invertebrate predators, including bacteria, fungi, nematodes, protozoa, mites, wasps, and flies (Riddick et al., 2009).

1.4 Microsporidia

Microsporidia are obligate, intracellular eukaryotic fungal pathogens found in terrestrial, marine, and freshwater ecosystems. They infect a wide variety of vertebrate and invertebrate hosts, including economically important silkworms, honeybees, shrimp, fish, and lady beetles (Han et al., 2020). The presence of microsporidia in lady beetles may result in unintentional importation and release of the pathogen when beetles are used for biological control.

In June of 1959, the microsporidian *Nosema hippodamiae* Lipa was found infecting convergent lady beetles, *Hippodamia convergens* Guérin-Méneville, collected from both Linden and Patterson, California. Each year, countless *H. convergens* are collected from overwintering

sites in California to be used for biological control of aphids on agricultural lands across North America (Riddick et al., 2009). Beetles may be infected with the microsporidium *Tubulinosema hippodamiae* and although they show no external symptoms of infection, the mid-gut and fat body are infected with the pathogen (Lipa and Steinhaus, 1959). Currently, *T. hippodamiae* is present in less than 1% of commercially available *H. convergens*, however, commercial suppliers often sell quantities of beetles from hundreds to hundreds of thousands. Therefore, thousands of infected beetles could be released each time *H. convergens* is used for biological control (Bjornson, 2008). The release of microsporidia-infected *H. convergens* (or other microsporidia-infected natural enemies) may result in the unintended dispersal of microsporidia, causing an increase in pathogen transmission and impacting the success of the biological control method and the integrity of native lady beetle communities already present in the region (Riddick et al., 2009).

Microsporidia can spread by horizontal transmission from one individual to another of the same generation (Han et al., 2020) when spores are released into the environment where they may be ingested through contaminated food or water. Ingested spores infect the gut epithelium of the host where they may spread to other tissues following infection. The more spores produced and released in the environment, the greater the opportunity of infecting a new host (Dunn and Smith, 2001).

Microsporidia can also be transmitted directly from infected parent to progeny, known as vertical transmission (Han et al., 2020). Vertical transmission is uniparental; the spores are passed from the female to the zygote through the egg cytoplasm, whereas male gametes are extremely small and rarely contribute to the cytoplasm of the zygote. Effective vertical transmission is dependent on host survival and reproduction (Dunn and Smith, 2001).

Some species of microsporidia under certain circumstances can alter sex ratios, which then alter population dynamics. The microsporidium *Thelohania californica*, which infects the Western Encephalitis Mosquito, *Culex tarsalis* Coquillet is a male-killing microsporidian. Such microsporidia are benign in female hosts but kill male mosquitoes to release their spores for horizontal transmission, which in turn increases the number of spores released in the environment (Kellen et al., 1965). Similarly, the microsporidian *Nosema granulosis* that infects the freshwater amphipod *Gammarus duebeni* Lilljeborg is a feminiser microsporidian. Feminiser microsporidia are benign in female hosts but convert genetic male hosts into functional, phenotypic females, which ultimately increases the vertical transmission of the pathogen to the next generation of hosts. Therefore, both male-killer and feminiser microsporidia may spread through host populations and alter sex ratios with the potential to drive a given population extinct due to a lack of males (Dunn and Smith, 2001).

Microsporidia have a two-part life cycle including a proliferative (developmental) phase which involves membrane-bound life stages that divide by merogony, asexual reproduction by binary or multiple fission, and a sporulation (transmission) phase whereby one or more spore types are produced for transmission and persistence in the environment (Han et al., 2020; Dunn and Smith, 2001).

Microsporidian spores contain a long, coiled polar filament that is attached to a mushroom-shaped anchoring disc. These unique structures are involved in spore germination, and they surround a single nucleus or pair of abutted nuclei (the diplokaryon) and reduced cytoplasmic organelles (Han et al., 2020). Microsporidia lack some organelles, including the Golgi apparatus and mitochondria, but contain remnants of these organelles (Han et al., 2020). Microsporidia have a highly reduced genome compared to other eukaryotes. The microsporidian

genome contains approximately 3000 protein-coding genes, and lacks almost all genes for functional ATP generation. They have highly reduced mitochondria (mitosomes) that lack normal mitochondrial function (Dunn and Smith, 2001, Han et al., 2020).

The infective form of the pathogen is an environmentally resistant spore, which is the only form of the pathogen that is extracellular and capable of persisting in the environment for months or longer under ideal conditions (Han et al., 2020). When the spore is ready to germinate, an inflow of water leads to an increase of pressure inside the spore which eventually ruptures the spore wall. This forces the long, coiled polar filament to eject, turning inside out to form the polar tube. The cytoplasm of the microsporidia, the sporoplasm, is pushed through the polar tube and either delivered to the surface of the host cell where it interacts with the cell membrane or is injected from the polar tube into the host cell cytoplasm directly (Keeling, 2009).

Following introduction into the host cell, the sporoplasm undergoes development into meronts, membrane-bound multinucleated forms that reproduce rapidly (Han et al., 2020). The plasma membranes of the meronts are in direct contact with the cytoplasm of the host cell where they parasitize the host cell organelles (Dunn and Smith, 2001). Next, the meronts develop into sporonts, then sporoblasts which are the developing spores, and finally into mature spores capable of infecting other host cells (Han et al., 2020).

Microsporidia infection of the host cell causes changes related to metabolic dependency because the microsporidian pathogen cannot grow or divide outside of the host cell. Due to their reduced mitosome, microsporidia lack certain metabolic pathways, including oxidative phosphorylation, electron transport, and the tricarboxylic acid cycle, and are unable to synthesize small molecules including amino acids and nucleotides. Therefore, microsporidia are dependent on host energy for pathogen growth and reproduction (Keeling, 2009). Following infection,

microsporidia often initiate the host cell to surround the exospore with mitochondria. The host cell mitochondria supply microsporidia with energy that is used for vegetative development and spore production. This ultimately results in a depletion of host energy that may have otherwise been used for growth and development (Keeling, 2009). For example, in local populations of *A. bipunctata* collected in Nova Scotia, Canada, the development of the microsporidian pathogen *Vairimorpha (Nosema) adaliae* results in prolonged larval development (Steele and Bjornson, 2012; Steele and Bjornson, 2014).

1.5 Plant Secondary Compounds

Plant secondary compounds are used for protection against predators, pathogens, and parasites (nematodes, fungi, bacteria, and insects) in plants (Price, 1977). These compounds are synthesized primarily for defence, which differs from primary compounds (nucleic acids, amino acids, carbohydrates, and fats), which are synthesized for normal growth, development, and reproduction. Secondary compounds from plants include terpenoids, alkaloids, and phenolics, which are further subdivided into flavonoids and non-flavonoids, such as tannins (Kabera, 2014). Essential oils are plant secondary compounds composed of a complex volatile mixture of alcohols, aldehydes, phenols, esters, ethers, and terpenes of varying concentrations (Barbieri and Borsotto, 2018).

Essential oils are present in low concentrations in the cell cytoplasm of localized plant secretory structures, including hairs or trichomes, epidermal cells, secretory ducts, secretory cavities, and resin ducts (Barbieri and Borsotto, 2018; Von Fraunhofer and Joshi, 2019). Essential oils are obtained from the leaves, stems, bark, seeds, fruits, roots, and flowers and are made available through the production of liquid extracts through solvent extraction or are the products of steam or dry distillation (Barbieri and Borsotto, 2018). An estimated three thousand essential

oils are known with at least 150 used commercially for the manufacture of cosmetics, fragrances, pharmaceuticals, and additives in food (Barbieri and Borsotto, 2018; Von Fraunhofer and Joshi, 2019).

The growth in production and sales of essential oils in the global market is driven by the increasing demand for natural and organic products primarily for pest control alternatives to synthetic pesticides, which are often associated with economic or regulatory environmental concerns. Many plant essential oils are highly toxic to insects and can inhibit moulting and respiration, reduce fecundity and growth, and disrupt cuticle development through fumigation and topical application (Digilio et al., 2008; Barbieri and Borsotto, 2018). For example, applying essential oils of fennel (*Foeniculum vulgare* Miller), basil (*Ocimum basilicum* L.), and anise (*Pimpinella anisum* L.) on the cayenne pepper plant (*Capsicum annum* L. var. *frutescens*) had effective insecticidal activity against the green peach aphids (*M. persicae*) without any side effects of phytotoxicity to the plant, even at high doses (Digilio et al., 2008).

Additionally, essential oils can reduce the fitness of a pest without causing lethal effects on insect metamorphosis and reproduction. Essential oils can act as antifeedants and repellants by introducing a toxin, an unpleasant taste, or by emitting an offensive odour. Some secondary compounds, especially tannins, may reduce the nutritional value of the plant. A given essential oil may act as an attractant to one insect and a repellant to another, with most herbivorous insects feeding on a small number of related plant species that belong to the same genus or family. The preferred host plants of a given herbivorous insect may share similar secondary compounds but be different in general morphology and anatomy (Harborne, 1993). For example, the anise swallowtail butterfly, *Papilio zelicaon L.*, which normally feeds on fennel will also feed on oranges in California orchards. This change in feeding preference is due to the presence of three

feeding attractants (anethole, estragole, and anisaldehyde) in the essential oils of the related families of Apiaceae (fennel), and Rutacea (orange) (Harborne, 1993).

In addition to the management of pests, essential oils are also practical for the management of fungi on crops. In tomato and potato plants, essential oils derived from oregano (*Origanum syriacum* L. var. *bevanii*), thyme (*Thymbra spicata* L. subsp. *spicata*), lavender (*Lavandula stoechas* L. subsp. *stoechas*), rosemary (*Rosmarinus officinalis* L.), laurel (*Laurus nobilis* L.), and fennel demonstrated *in vitro* antifungal activities against the late blight pathogen, *Phytophthora infestans* Montagne de Bary. All of these essential oils were effective for inhibiting the growth of the pathogen, but oregano is the most effective at preventing sporangium formation, followed by thyme, fennel, lavender, rosemary, and laurel, respectively. Furthermore, *P. infestans* hyphae grown on media to which essential oils were added exhibit morphological alterations when compared to normal mycelia including shrivelled hyphal aggregates with reduced diameters and thinning of the hyphal wall (Soylu et al., 2006).

Plant essential oils of anise, fennel, and marjoram (*Origanum majorana* L.) in honey have also demonstrated *in vivo* antifungal activity against the microsporidia pathogen *Nosema ceranae*, in the honeybee, *Apis mellifera* L., as an alternative treatment for Colony Collapse Disorder (CCD) caused by microsporidia. Anise honey was the most effective at reducing the percentage of infected bees, followed by fennel, then marjoram (Darwish, 2021). Additionally, the antioxidant activity of the flavonoids present in aromatic honey is highest in the anise honey, followed by fennel, then marjoram, which contributes to the reduction of microsporidia infection in honeybees (Darwish, 2021).

1.6 Sweet Fennel: Foeniculum vulgare var. dulce Miller

The use of fennel essential oil for pest and fungal management is associated with low environmental persistence and minimal health risks for humans and other animals. Fennel essential oil is also readily available and affordable, and as a naturally derived compound it has gained wide public acceptance (Digilio et al., 2008; Pavela, 2018). Fennel, *Foeniculum vulgare* Miller, is a perennial herbaceous plant belonging to the Apiaceae family. The aromatic plant is widespread in the Mediterranean and Central Europe and is cultivated throughout both temperate and tropical regions. There are three varieties of fennel, including bulbing or Florence fennel, *Foeniculum vulgare* var. *azoricum* Miller, sweet fennel, *Foeniculucm vulgare* var. *dulce* Miller, and bitter fennel, *Foeniculum vulgare* var. *vulgare* Miller. Bitter fennel can be distinguished from sweet fennel by the bitter aroma of its seeds and the presence of a stronger root, which allows the plant to better resist colder temperatures (Patrolea and Teodor, 2020). In addition, sweet fennel has heavier and less abundant flower inflorescences (umbels) than bitter fennel (Patrolea and Teodor, 2020). However, all parts of the fennel plant are used to produce commercially available essential oils with the above-ground portion of the plant providing high yields at an affordable price (Pavela, 2018).

The antioxidant, antispasmodic, anti-inflammatory, analgesic, and diuretic effects of fennel essential oil make it a common additive to perfumes, toothpaste, soaps, and herbal medicines (Pavela, 2018). The essential oil content of fennel varies and is dependent on the variety and origin of cultivation (Patrolea and Teodor, 2020). Fennel essential oil consists predominantly of oxygenated monoterpenes including *trans*-anethole, fenchone, and estragole, and hydrocarbon monoterpenes including α-pinene and limonene. The complex monoterpenoid composition of fennel essential oil is proposed to contribute to the development of safe, fully

biodegradable, and effective control of targeted pests, pathogens, and parasites (nematodes, fungi, bacteria, and insects) with lower development of resistance (Lacotte et al., 2023; Digilio et al., 2008; Price, 1977).

The monoterpene compounds in fennel essential oil are weakly repellant against the pea aphid, *Acrythosiphon pisum* Harris, with high concentrations of *trans*-anethole and estragole contributing to the repellant effect, and low concentrations of α-pinene and limonene contributing to an attractive effect (Lacotte et al., 2023). Aphids can detect these different volatile compounds through their antennae, which are equipped with olfactory receptor neurons that induce a behavioural response (of either flight or forage) to the odour. The essential oil released from the plant and the potential lack of available alternative food sources could impair the insect response and could result in the essential oil having a repellant or attractive effect on one species but eliciting a different response on another (Lacotte et al., 2023). For example, fennel essential oil provides effective insecticidal activity against *M. persicae*, without causing significant mortality against the non-targeted Asian ladybeetle, *Harmonia axyridis* Pallas (Pavela, 2018).

Under semiarid field conditions of Southeastern Europe, where food for lady beetles may become scarce in the summer, the continued presence of fennel and other flowering plants from June to October provides alternative food resources of nectar and pollen (Hodek and Honěk, 1996). In addition, the odour of fennel is attractive to *A. bipunctata* for oviposition sites (Iperti and Prudent, 1986). The ability of fennel essential oil to attract *A. bipunctata* combined with its utility for pest and fungal management, indicates the potential for fennel essential oil to manage microsporidia infection in *A. bipunctata*. The use of essential oils from fennel may contribute to the control of fungal pathogens, such as microsporidia, when administered to *A. bipunctata* similar to how this plant family Apiaceae and many others (such as Rosaceae, Brassicaceae,

Asteraceae, and Poaceae) produce phytoalexin compounds for protection in response to fungal attacks in nature (Harborne, 1997). Therefore, the objective of this research is to study the effects of sweet fennel, *Foeniculum vulgare* var. *dulce*, essential oil on the microsporidian pathogen *Vairimorpha (Nosema) adaliae* in the two-spotted lady beetle, *A. bipunctata*. The effects on larval development, survival, and sex ratios of newly eclosed beetles of infected individuals were investigated. Understanding the role of secondary plant compounds on pathogens in natural enemies will help to ensure that beneficial insects, such as lady beetles, remain effective biological control predators as an alternative to synthesized pesticides.

2. Materials and Methods

2.1 Caring for Stock Adalia bipunctata

Uninfected and *Vairimorpha*-infected *Adalia bipunctata* stock beetles were reared in the laboratory from previously established colonies within environmental test chambers (Sanyo Scientific MLR-350H; 16:8 L:D; 25°C:20°C). *A. bipunctata* adults were reared in 120 ml clear polyethylene cups with a 22 mm diameter hole on the side that was covered with fine cloth mesh to allow for ventilation. The cups were washed prior to use with soap and water, rinsed, soaked in 10% bleach solution for 15 minutes, rinsed again, and left to air dry.

A. bipunctata adults were provided distilled water through a moistened half piece of non-sterile cotton roll (Advantage Plus; Crosstex International, NY) and fed an artificial diet consisting of equal parts of Lacewing and Ladybug Food (Planet Natural, MT) and organic pure honey (President's Choice; Loblaws, ON). A small portion of the artificial diet was spread onto the edge of the mesh on the inside of the cup. Throughout larval development, and when required to lay eggs, *A. bipunctata* were also fed green peach aphids, *Myzus persicae*, that had been reared in a separate environmental test chamber on nasturtium, *Tropaeolum minus L.*, (Dwarf Jewel

Mixed Double; Stokes Seeds, ON) grown from seed in growing medium (PRO-MIX; Premier Horticulture Ltée Ltd., QC).

2.2 Preparation of Sweet Fennel Solutions

In a 100 ml graduated cylinder, 10 ml of Sweet Fennel Tincture (St. Francis Herb Farm Inc., ON) was added using a 10 ml serological pipet. The tincture consisted of certified organic sweet fennel seeds, *Foeniculum vulgare* subsp. *vulgare* var. *dulce*, distilled water, and alcohol. The sweet fennel was then diluted with 90 ml of distilled water to create a 10% sweet fennel solution, which was stored in a refrigerator (3ºC; Sanyo Scientific Medicool MPR-414F) when not in use. This process was repeated for a 20% sweet fennel dilution (20 ml of sweet fennel tincture in 80 ml of distilled water) and a 30% sweet fennel dilution (30 ml of sweet fennel tincture in 70 ml of distilled water).

2.3 Trial Set Up

Ten randomized mating pairs of both uninfected and *V. adaliae*-infected *A. bipunctata* adults (n = 20 individuals total) were isolated in 120 ml polyethylene cups with one male and one female in each cup. Mating pairs were fed green peach aphids daily and distilled water was provided on half a piece of cotton roll. The underside of each lid was lined with a piece of 55 mm diameter filter paper (Whatman; Cytiva, CA) for the eggs to be laid on. Mating pairs were observed daily and replenished with aphids and distilled water. Newly laid eggs were collected every day and placed in separate 120 ml polyethylene cups with the date and mating pair parent identification number recorded. Once the eggs hatched into larvae, a quarter piece of cotton roll was soaked with distilled water and added to the cup. Newly hatched larvae were not given diet

or aphids (no food) for 24 hours to increase their likelihood of consuming a *V. adaliae*-infected egg later on for transmission of the microsporidian pathogen (Fletcher and Bjornson, 2018).

After 24 hours had lapsed, each uninfected *A. bipunctata* larva was isolated into a 50 mm diameter polycarbonate Petri dish (Millipore Corp., MA). Larvae were handled with a fine paintbrush (White Taklon; Heinz Jordan & Company Ltd., ON). A mesh-covered 30 mm diameter hole in the Petri dish lid allowed for air circulation and prevented larval escape. Prior to use, the Petri dishes had been washed with soap and water, rinsed, soaked in 10% bleach solution for 15 minutes, rinsed again, and left to air dry.

Each *A. bipunctata* larva was provided with a single *V. adalia*-infected *A. bipunctata* egg that had been collected from different *V. adalia*-infected mating pairs. To ensure the presence or absence of *V. adaliae* in the infected and uninfected mating pairs, respectively, the parents and some of the eggs from each pair were smeared and stained with 5% Giemsa (pH 6.9, Sigma-Aldrich, MO). These specimens were then examined at 40x magnification with a compound light microscope (Olympus CX23) for *V. adaliae* spores. When present, *V. adaliae* spores are transmitted horizontally (Fletcher and Bjornson, 2018). A single infected egg was placed on a moistened 6 mm diameter filter paper disc. Eggs that were used in this study were 24 hours old, which prevented the eggs from hatching before the test larvae could consume them. The mating pair parent identification number and the source of the infected egg that had been provided were recorded.

Each *A. bipunctata* test larva was assigned randomly to four treatments and individuals were either provided with water (control), or a solution of sweet fennel (10%, 20%, or 30%) saturated on a quarter piece of cotton roll using separate disposable pipets per treatment to prevent contamination. For each fennel group, five individuals were isolated each day for five

days, yielding 25 individuals per fennel treatment. For the control group, five individuals were isolated each day for five days with 10 individuals prepared on the sixth day, yielding 35 individuals for the control treatment. An additional sixth day in the control group was needed due to a large number of larval deaths occurring before they were able to consume the *V. adaliae*-infected egg. The trial was repeated a second time in the same manner, but without an additional sixth day in the control group (50 individuals per fennel treatment and 60 individuals for the control treatment; total n = 210), as the majority of larva did not die before consuming the *V. adaliae*-infected egg in the repeated trial. Larvae were reared within one environmental chamber for the duration of the trial.

2.3.1 Larval Development and Mortality

The filter paper discs were examined under a stereomicroscope (Zeiss Stemi 2000-c) 24 hours after the *V. adaliae*-infected egg was introduced to confirm an egg was consumed by each larva. Filter paper discs were removed from Petri dishes for those larvae that had consumed the egg but were left in the dish for an additional 24 hours for larvae that had not yet consumed the egg. Larvae that had consumed the *V. adaliae*-infected eggs were provided with a diet of green peach aphids and the cotton roll was replenished with distilled water or the appropriate fennel solution depending on the treatment group. The larvae were observed daily, were provided with aphids, and replenished with distilled water or fennel solution (depending on treatment) until they eclosed as adults. The stage of development was recorded daily for each larva to determine the larval development time in days. Eventually, larvae began to curl and remained immobile as they entered the pre-pupal stage. A larval moult was observed by the presence of a larval exuvia in the Petri dish. Larvae, pupae, or adults that had died during the trial were also recorded to determine the mortality rate. Data was excluded for larvae that did not eat both eggs after 48 hours.

2.3.2 Sex Ratio

After *A. bipunctata* larvae emerged as adults, they were provided with distilled water or fennel solution, depending on the treatment, and left alone with no food for 24 hours. The following day, each adult was placed into a 100 mm diameter polystyrene Petri dish (Fisherbrand; Fisher Scientific, ON) where it was examined under a stereomicroscope. The sex of each adult was determined by examining the morphology of the seventh and eighth abdominal sternites (see Plate XI; Hodek, 1973).

Sexed adults were dissected under the stereomicroscope using a 4 mm straight-blade dissecting knife (Fine Science Tools Inc., DE). The determined sex of the emerged adult was confirmed by identifying internal sex organs (see Plate XI; Hodek, 1973). All individuals, including the sexed adults and larvae or pupae that died prematurely, were individually smeared onto labelled microscope slides for *V. adaliae* infection status and spore counts and left to air dry.

2.4 Staining Procedure

Smeared preparations were placed into a glass slide rack in a rectangular, glass staining dish. Using a spring wire handle to transfer the slide rack between nine different staining dishes, slides were fixed in 100% methanol (Commercial Alcohols, Greenfield Speciality Alcohols Inc., ON) for 10 min, rinsed in tap water (10 min), and stained with 5% Giemsa solution (pH 6.9, Sigma-Aldrich, MO) for 2 h. Slides were then dehydrated in a series of anhydrous ethyl alcohol (Commercial Alcohols; Greenfield Speciality Alcohols Inc., ON) solutions from 70% (3 min), 80% (3 min), 90% (3 min), 95% (3 min) and 100% (3 min), ending in xylene (10 min; Fisher Scientific, NJ). Stained slides were mounted with Permount mounting medium (Fisher Chemical; Fisher Scientific, NJ). Slides were placed within a fume hood overnight to dry fully. Afterwards,

the stained specimens were examined with a compound light microscope at 40x magnification for the presence or absence of *V. adaliae* spores.

2.5 Vairimorpha adaliae Spore Counts

Stained adult specimens confirmed with *V. adaliae* spores were examined with a compound light microscope (Zeiss Imager.AI AXIO) using oil immersion (Zeiss 518F Immersol) at 100x magnification. A total of five randomly chosen areas (total area: 120 μm^2) of each prepared slide were photographed (Zeiss Axiocam 208 Color Camera) and observed in the Zen microscopy software (Zeiss Zen 3.8) to visually count the number of spores in each image.

2.6 Data Analysis

The data for the repeated trials was combined and analyzed in R Version 4.2.3 (R Core Team, 2023) using R Studio Version 2023.12.0.369 (Posit Team, 2023). For analysis of both larval development and *V. adaliae* spore count data, a Sapiro-Wilk Normality Test was used to determine if the data sets were normally distributed. As the data sets were not normally distributed, a Kruskal-Wallis Test was used to analyze the difference in mean spore counts between treatment groups and the difference in mean larval development time for all individuals together and separated by sex, between all treatment groups. In addition, a Wilcoxon Signed Rank Test was used to analyze the difference between female and male mean development time in each treatment group when compared to each other. Larval development data and *V. adaliae* spore count data obtained were visualized separately using the packages ggplot2 (Wickham, 2016) and qiime2R (Bisanz, 2018) for data manipulation and visualization, and viridis for colour-blind-friendly palettes (Garnier et al., 2023).

Mortality rate was calculated as percent larval and pupal death divided by the total sample size of each treatment for the repeated trials. A Chi-Square (χ^2) Test was used to determine if there was a significant association between fennel concentration and mortality rate, as well as if there was a significant association between fennel concentration and sex ratio of newly-emerged adults. Larvae that did not consume the *V. adaliae* infected egg or did not complete development by Day 22 of the trials were excluded from all data analyses.

3. Results

3.1 Larval Development and Mortality

The mean larval development time of *A. bipunctata* was comparatively similar between all treatment groups and did not differ significantly (H = 2.1714, *df* = 3, p = 0.5376; Figure 1A and Table 1). However, a greater number of larvae developed fully and eclosed as adults in the control (n = 31) and 10% fennel treatment (n = 33) when compared to the 20% (n = 25) and 30% fennel (n = 27) treatments. In addition, larvae from the 30% fennel treatment had the highest mean development time (17.67 ± 0.29 days), followed by the control and 20% fennel (17.48 ± 0.32 days, 17.48 ± 0.30, respectively), with the lowest mean development time for larvae in the 10% fennel treatment (17.12 ± 0.28 days).

Female and male larval development times between all treatment groups did not differ significantly (W = 1, p = 0.25, ♀: H = 1.7327, *df* = 3, p = 0.6297, ♂: H = 1.5009, *df* = 3, p = 0.6821; Figure 1B). The mean larval development for males tended to be shorter than females in the control (♂: 17.18 ± 0.58 days, ♀: 17.67 ± 0.43 days), and in those treated with 10% fennel (♂: 16.93 ± 0.38 days, ♀: 17.28 ± 0.42 days) and 30% fennel (♂: 17.44 ± 0.36 days and ♀: 18.00 ± 0.49 days). In contrast, females had a shorter larval development time than males in the 20% fennel treatment (♀: 17.38 ± 0.38 days, ♂: 17.67 ± 0.50 days).

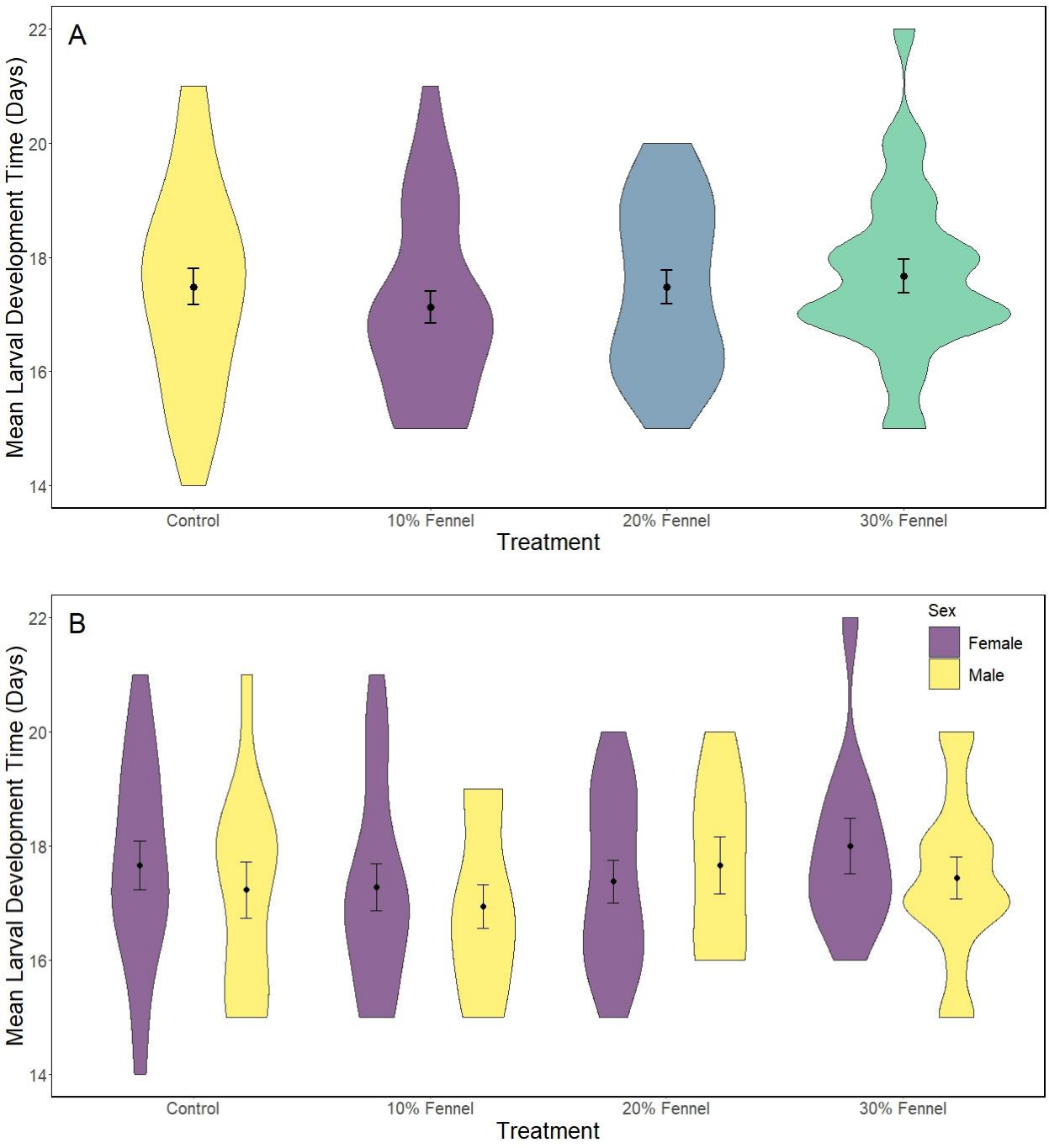

Figure 1. Mean larval development time in days for *Vairimorpha adalia*-infected *Adalia bipunctata* (**A**) per treatment and (**B**) per treatment separated by sex of female (purple) and male (yellow) for individuals that were provided either water (control) or different concentrations of fennel essential oil (10%, 20%, and 30%). Error bars represent standard error. Larval development per treatment: Kruskal Wallis (H = 2.1714, *df* = 3, p = 0.5376). Larval development for females and males per treatment: Kruskal-Wallis (♀: H = 1.7327, *df* = 3, p = 0.6297, ♂: H = 1.5009, *df* = 3, p = 0.6821). Larval development time between females and males: Wilcoxon Test (W = 1, p = 0.25). Means did not differ significantly (P > 0.05).

Percent larval mortality between the treatment groups did not differ significantly ($\chi 2$ = 1.4562, df = 3, p = 0.6924; Table 1). Percent larval mortality was highest in the 20% fennel group (30.56%) with a total of eleven deaths. In comparison, the lowest larval mortality was in the control (20.51%) and 30% fennel group (20.59%) but higher in the 10% fennel group (21.43%) with a total of eight, seven, and nine deaths, respectively.

3.2 Sex Ratio

The sex ratio for newly eclosed adults did not differ significantly between the treatment groups (χ^2 = 3.1263, *df* = 3, p = 0.3726; Table 1). Sex ratio was comparatively the same between the control (18♀: 13♂) and the 30% fennel group (11♀: 16♂) however, five additional females were recorded in the control and five additional males were recorded in the 30% fennel treatment. The greatest difference in sex ratio was among individuals from the 20% fennel group (16♀: 9♂) where there were an additional seven males compared to females. In contrast, the least difference in sex ratio was among individuals from the 10% fennel group (18♀: 15♂) where there were an additional three females compared to males. Overall, there were more females (n = 63) than males (n = 53) that fully eclosed as adults among all treatment groups.

Table 1. Adult sex ratio, mean larval development time in days (mean ± standard error), and percent larval mortality (%) for *Vairimorpha adaliae*-infected *Adalia bipunctata* that were provided with either water (control) or different concentrations of fennel essential oil (10%, 20%, and 30%).

	Sex Ratio	Larval Development		Larval Mortality	
Treatment (total *n*)	(♀: ♂)	*n*	Mean Days ± SE	*n*	Mortality (%)
Control (39)	(18:13)	31	17.48 ± 0.32	8	20.51
10% Fennel (42)	(18:15)	33	17.12 ± 0.28	9	21.43
20% Fennel (36)	(16:9)	25	17.48 ± 0.30	11	30.56
30% Fennel (34)	(11:16)	27	17.67 ± 0.29	7	20.59

Larval Development: Kruskal-Wallis (H = 2.1714, *df* = 3, p = 0.5376). Means did not differ significantly (p > 0.05). Sex Ratio: Chi-square (χ^2 = 3.1263, *df* = 3, p = 0.3726). Larval Mortality: Chi-square (χ^2 = 1.4562, *df* = 3, p = 0.6924). Sex ratios and mortality did not differ significantly (p > 0.05).

3.3 Vairimorpha adaliae Infection and Spore Counts

Mating pairs from uninfected *A. bipunctata* stock beetles that were used to rear the uninfected test larvae were confirmed to lack *V. adaliae* spores. In addition, the presence of *V. adaliae* spores was detected in both the mating pairs from infected stock beetles and in eggs from the same clutches that had been used to feed the test larvae. *V. adaliae* spores were detected in all individuals from all treatment groups that were fed infected eggs.

Mean *V. adaliae* spore counts between all the treatment groups did not differ significantly (H = 7.7706, df = 3, p = 0.51, Figure 2). Control *A. bipunctata* adults had the lowest mean *V. adaliae* spore count (7.95 ± 0.86 spores/μm^2) followed by those from the 10% fennel group (8.93 ± 1.36 spores/μm^2). In comparison, higher spore counts were observed in the 20% fennel and 30% fennel groups (12.83 ± 2.33 spores/μm^2 and 12.58 ± 2.23 spores/μm^2, respectively), with

individuals from the 20% fennel group having the highest spore counts among all treatment groups. Overall, percent spore load was increased by 12.83%, 61.33%, and 58.24% in the individuals fed 10%, 20%, and 30% fennel concentrations, respectively.

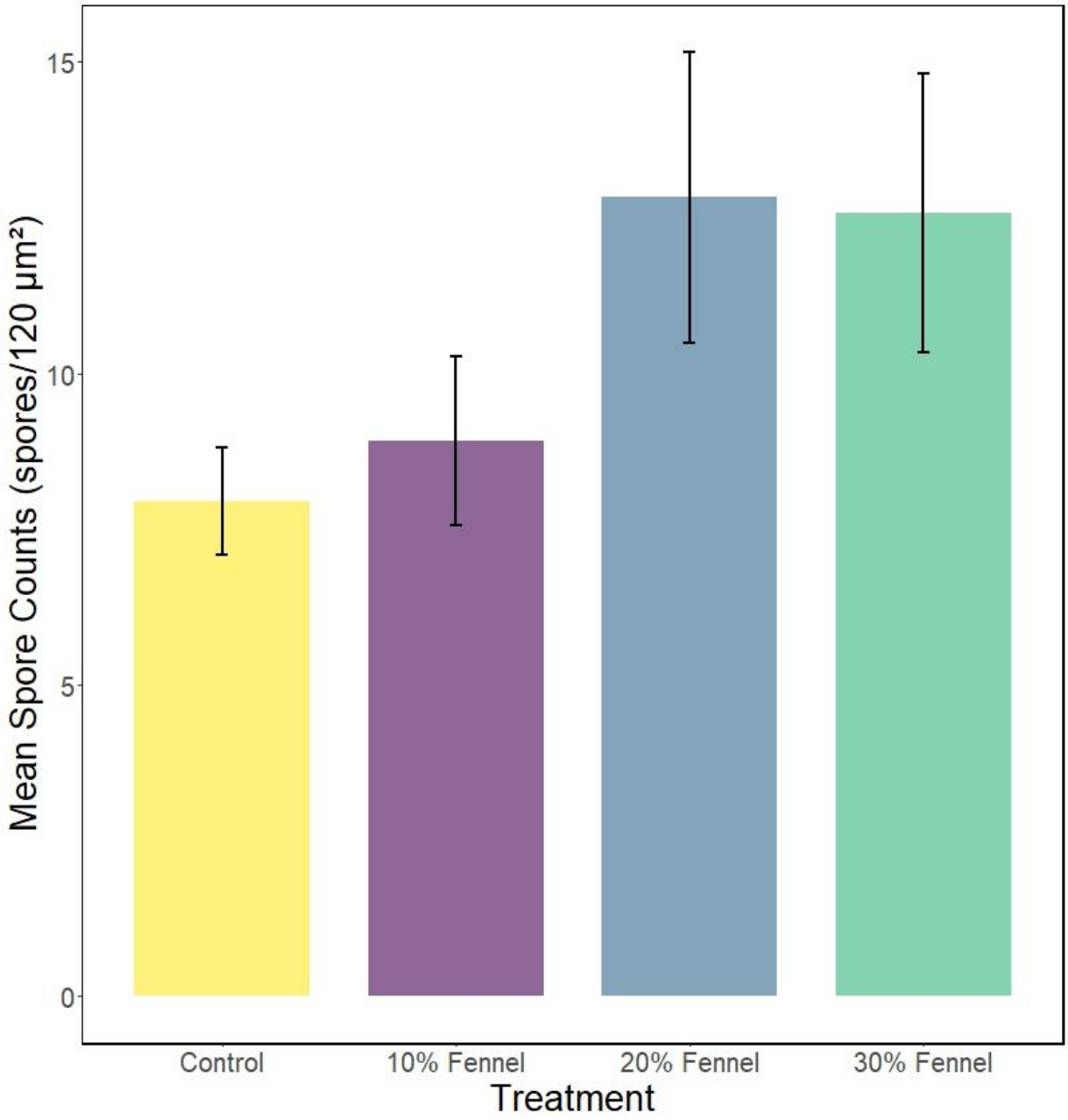

Figure 2. Mean spore count (spores/120 µm^2) for *Vairimorpha adalia*-infected *Adalia bipunctata* that were provided with either water (control) or different concentrations of fennel essential oil (10%, 20%, and 30%). Error bars represent standard error. Kruskal-Wallis (H = 7.7706, df = 3, p = 0.51). Means did not differ significantly (p > 0.05).

4. Discussion

Microsporidia are spore-forming, fungal pathogens that cause chronic, debilitating, and generally sublethal diseases in many beneficial insects (Bjornson and Oi, 2014; Steele et al., 2023). Due to economic and environmental concerns, there is a high demand for natural and organic products as an alternative to synthetic chemicals for the treatment of these pathogens (Barbieri and Borsotto, 2018). Plant secondary compounds, such as essential oils, are used by plants in nature for protection against predators, pathogens, and parasites (nematodes, fungi, bacteria, and insects) (Price, 1977), and these compounds should be examined for their potential to control microsporidia in beneficial insects.

Sweet fennel essential oil has been used in previous studies (Digilio et al., 2008; Lacotte et al., 2023; Pavela, 2018; Soylu et al., 2006; Darwish 2021) for pest and fungal management without causing adverse effects or significant mortality to non-target organisms. The complex monoterpenoid composition of fennel essential oil (composed of *trans*-anethole, fenchone, estragole, α-pinene, and limonene) is proposed to contribute to its effective control of pests and pathogens with lower development of resistance, along with being safe and fully biodegradable (Lacotte et al., 2023; Digilio et al., 2008; Price, 1977). However, the use of fennel essential oil has not yet been investigated for the management of the microsporidium *Vairimorpha adaliae* in the two-spotted lady beetle, *A. bipunctata*, which was the focus of this study.

4.1 Effects of Sweet Fennel on Vairimorpha adaliae

Egg cannibalism is considered part of the normal foraging behaviour of ladybeetles and feeding on both eggs and larvae is common in *A. bipunctata* (Steele and Bjornson, 2012, Steele and Bjornson, 2019). Microsporidian spores are observed in adults when they are fed infected eggs (horizontal transmission) as first or second-instar larvae (Saito and Bjornson, 2008). In this

study, horizontal transmission of *V. adaliae* in *A. bipunctata* occurred with 100% efficiency as all individuals examined were infected at the end of the trial. These results are consistent with those reported in previous studies (Steele and Bjornson, 2012; Steele and Bjornson, 2019). The addition of sweet fennel essential oil did not affect horizontal transmission efficiency within *A. bipunctata* larvae, as oral administration of the essential oil was provided after the infected egg was consumed by an uninfected larva.

In addition, fennel essential oil did not affect *V. adaliae* spore loads, although a notable increase of mean spores (heavier infection) was observed in the two highest (20% and 30%) fennel treatments. These results differ from a previous study (Darwish, 2021) where oral administration of fennel honey combined with water at three different concentrations (20%, 40%, and 60%) resulted in a significant reduction of *Nosema cerenae* infection in honeybees (*Apis mellifera*) in the highest (60%) fennel treatment (Darwish, 2021). This difference may be due to the use of fennel essential oil in our study instead of fennel honey.

Honey contains secondary phenolic compounds, such as high amounts of flavonoids that contribute to the antimicrobial properties that inhibit the growth of several pathogenic bacteria and fungi (Darwish, 2021; Bjornson et al., 2022). The role of antimicrobial properties provided by honey against *V. adaliae* infection in *A. bipunctata* is seen in a previous study by Bjornson et al. (2022) who found that providing manuka honey combined with water at three different concentrations (5%, 10%, and 15%) resulted in a notable reduction of mean spores (lighter infection) in the highest (15%) manuka honey treatment. The combination of other antimicrobial compounds may show a synergistic effect (the interaction and cooperation of two substances that produce a greater effect combined than the sum of their separate effects) with enhanced effectiveness (Rafieian et al., 2023). Therefore, the additional properties of the honey along with fennel used by Darwish (2021) may provide a combined treatment for the microsporidian

infection that did not occur with the use of fennel essential oil in this study. Future studies may focus on examining the synergistic effects of both antimicrobial honey and fennel on the microsporidian infection in beneficial lady beetles.

Further discrepancies between Darwish (2021) and other studies using fennel essential oil for pest and fungal management (Digilio et al., 2008; Lacotte et al., 2023; Pavela, 2018; Soylu et al., 2006; Darwish 2021) may be due to variation in the amounts of each component present. According to Cetin et al. (2010), anethole is the main component responsible for the antimicrobial activity of fennel essential oil. The amount of anethole present can vary depending on the conditions of farming, season changes, site of collection, processing, and storage conditions (Rafieian et al., 2023). In addition, the extracts of fennel flowers compared to other parts of the plant have been cited to show better antibacterial properties (Rafieian et al., 2023). This may account for variation in the results of this study, as the fennel essential oil used was derived from the seeds of the plant.

In general, it is known that insects have varying sensitivities to plant secondary compounds where a given essential oil may act as an attractant to one insect and a repellant to another (Harborne, 1993). Additionally, different bacterial and fungal species have varying sensitivities to fennel essential oil in particular, with certain species more sensitive (*Bacillus subtilis, Aspergillus* spp., and *Trichophyton* spp.) while others are more resistant (*Escherichia coli* and *Pseudomonas aeruginosa*) (Rafieian et al., 2023). Microsporidian species likely have the same variation in sensitivity exhibiting discrepancies in their response to fennel essential oil. Further studies should include an examination of fennel essential oil as an antifungal treatment examining other species of microsporidia that infect ladybeetles.

4.2 Effects of Sweet Fennel on Adalia bipunctata

Larval development time in lady beetles is highly dependent on variations in environmental conditions, including temperature, photoperiods, food quality and quantity (Mishra and Omkar, 2012). However, when these variables are kept constant in laboratory conditions, lady beetles tend to spend approximately the same proportion of time devoted to development with a similar ratio of slow and fast developers (Dixon, 2000; Mishra and Omkar, 2012). This is shown in the relatively regular distribution around the mean larval development time in the control treatment (Figure 1A). However, by incorporating fennel essential oil as a fluctuating environmental factor between treatment groups, individual larvae may respond differently through their development time. An individual beetle may complete development earlier or later compared to the mean development time in response to the progression and degree of microsporidian infection or their response to an increased dose of fennel essential oil. This is shown by a shift in distribution around the mean with increasing fennel concentrations where low doses result in a slight variation from the mean (10% fennel), followed by a bimodal distribution (20% fennel) and a prominent irregular distribution (30% fennel) around the mean of *A. bipunctata* development times (Figure 1A).

Larval development time is also dependent on the sex of the beetle where there is a female-biased ratio of slow-developers (Mishra and Omkar, 2012). The trend is associated with an improvement in adult fitness as females of slow-developing larvae can produce and lay more eggs than fast-developing larvae (Mishra and Omkar, 2012). This is shown in the control treatment (Figure 1B) where male larvae develop earlier than female larvae. Considering the addition of fennel essential oil as a fluctuating variable in otherwise constant laboratory conditions, the male development time range seems to shorten but the distribution of individual beetle variation around the mean is unaffected and depicts a relatively regular distribution in

lower fennel concentrations (10% and 20%) (Figure 1B). However, in a higher concentration of fennel (30%), males depict a clustered distribution around the mean where earlier (closer to 16 days) and later (closer to 18 days) development times occur in more individuals within the sample size than at the mean (17 days) (Figure 1B). However, greater variations in female distribution around the mean seem to occur in higher concentrations of fennel (30%) (Figure 1B) This may indicate that female individual beetles are more variable in their response to a stressful environmental condition (fennel essential oil) due to the energy needed for optimal reproduction of high quality and quantity of eggs observed in slow developing larvae (Mishra and Omkar, 2012).

Prolonged larval development is typical of microsporidiosis in several lady beetle species, including *Coccinella septempunctata, Harmonia axyridis, Hippodamia convergens,* and *Adalia bipunctata* (Saito and Bjornson, 2008; Steele and Bjornson, 2012). Larvae with delayed development are more susceptible to cannibalism and predation for an extended period (Steele and Bjornson, 2019). Saito and Bjornson (2008) reported that larval development time for microsporidia-infected *A. bipunctata* was significantly delayed by about one day when compared to uninfected larvae. In our study, developmental time was between 17 to 18 days for infected larvae which aligns with Steele and Bjornson (2012) and Steele and Bjornson (2019) who found that the mean developmental time was 18.07 days and 17.92 days, respectively, for infected *A. bipunctata* larvae fed a similar diet of green peach aphids daily. The addition of sweet fennel essential oil in this study did not affect mean larval development time overall nor between male and female beetles.

The prolonged larval development time is caused by a depletion of energy in the host due to infection by the microsporidian pathogen (Steele. 2020). Microsporidia have highly reduced mitochondria (mitosomes) that lack normal mitochondrial function (oxidative phosphorylation,

electron transport, and tricarboxylic acid cycle) (Han et al., 2020; Luo et al., 2021) Therefore, they are strictly dependent on host energy for intracellular growth and reproduction (Keeling, 2009). Some species of microsporidia (*Encephalitozoon cuniculi*, *E. intestinalis*, and *E. hellem*) can increase the redistribution and localization of host mitochondria around the exospore to be used for their own energy (Bailey et al., 1999). In this study, *A. bipunctata* larvae treated with 20% and 30% fennel had similar development times but were able to withstand heavier infections than control larvae that were provided water only (no fennel essential oil). This may indicate that additional energy is somehow provided to these larvae to offset the effects of *V. adaliae* through an increase in the rate of metabolic processes, or by the localization and redistribution of host mitochondria. However, further investigations of *A. bipunctata* tissue morphology and physiology are needed to substantiate this speculation.

Sweet fennel essential oil did not affect overall larval mortality which suggests that it is safe to use on *A. bipunctata* and aligns with Digilio et al. (2008) and Pavela (2018) who found that fennel essential oil had successful insecticidal activity against green peach aphids without having any phytotoxicity or adverse effects to non-target ladybeetles (*Harmonia axyridis*). Although sweet fennel essential oil can effectively reduce or eliminate aphid populations through topical application (Digilio et al., 2008; Pavela 2018), the potential death of aphids in our study was not examined but it was unlikely to have an impact on the results. Suboptimal diets are known to increase susceptibility and virulence to a pathogen for many insects as indicated by Steele and Bjornson (2019) who found that *V. adaliae*-infected *A. bipunctata* provided an irregular diet had a further delayed larval development and increased spore loads (heavier infection). However, by providing isolated *A. bipunctata* with a diet of green peach aphids daily and the fennel essential oil at a reduced concentration on a localized piece of cotton, the potential death of aphids and subsequent starvation period of the beetle was reduced. Additionally, if the

death of aphids did occur, larvae would still have fed on the aphids as dead (frozen) aphids have been used previously to rear *A. bipunctata* under laboratory conditions.

Some species of microsporidia under certain circumstances can alter sex ratios of invertebrates (Kellen et al., 1965; Dunn and Smith, 2001) but it has not been reported in *A. bipunctata*. In this study, sweet fennel essential oil had no effect on sex ratios of newly eclosed adult beetles which is comparable to previous studies (Steele and Bjornson, 2012; Steele and Bjornson, 2019) that observed no difference in sex ratios of *A. bipunctata* when infected with a microsporidian pathogen.

4.3 Applications for Future Research

Microsporidian infection of *V. adaliae* in *A. bipunctata* results in prolonged larval development, but does not affect adult fecundity, longevity or sex ratios (Steele and Bjornson 2012; Steele and Bjornson 2014). In this study, the use of sweet fennel essential oil to treat microsporidiosis in *A. bipunctata* had a notable heavier infection in increased concentrations, but no effect on mortality, sex ratios, or prolonged larval development caused by the pathogen. However, adult fecundity was not investigated. Iperti and Prudent (1986) indicated that the odour of fennel is attractive to *A. bipunctata* for oviposition. Therefore, an examination of the effects of sweet fennel on *A. bipunctata* fecundity, such as the viability and quantity of eggs produced as well as the implications these effects have on the microsporidian pathogen (infection within the eggs and vertical transmission efficiency) throughout consecutive generations, is worth future investigation.

In addition, the effects of sweet fennel on *A. bipunctata* longevity and the ability of adult beetles to survive with heavier infection were not investigated. A limitation of this study is that spore counts (*V. adaliae* infection) were determined only once throughout an individual's

lifetime as a destructive sampling technique (dissection of the adult beetle) was employed for analysis. The spores of *V. adaliae* infect various tissues involved with energy-dependent processes including reproduction (reproductive organs), movement and sense (flight muscles and ventral nerve cord), intermediate metabolism (fat body), and excretion (midgut and hindgut consisting of Malpighian tubules, ileum, and colon) (Saito, 2008; Steele and Bjornson, 2014). In our study, it was speculated that *A. bipunctata* given higher concentrations of fennel essential oil demonstrate a metabolic response to heavier infections of *V. adaliae* by providing additional energy to the host beetle to withstand the effects of the pathogen and complete development at the same rate as control beetles.

Therefore, as the midgut and hindgut are known to be infected (Saito, 2008; Steele and Bjornson, 2014), spores may be detected in the fecal matter of infected *A. bipunctata* for non-destructive sampling to determine the changes in infection status and the impacted energy-dependent processes that may occur throughout their lifetime (based on age), as well as through consecutive generations. However, previous studies using other ladybeetles (*H. convergens)* observed minimal success in microsporidian spores detected in fecal samples (Joudrey, 2006). Future studies in *A. bipunctata* are worth investigating.

4.4 Implications in Biological Control

Natural enemies, such as lady beetles, are widely available and used for biological control of crop pests (Caltagirone and Doutt, 1989). Their susceptibility to a variety of parasitoids and pathogens (bacteria, nematodes, protozoa, mites, wasps, flies, and fungi) specifically microsporidia, can cause chronic, debilitating and sublethal disease (Riddick et al., 2009; Bjornson and Oi, 2014; Steele et al., 2023). Their presence can result in the unintentional importation and release of the parasitoids or pathogens resulting in increased transmission and

impacting both the success of the biological control method and the integrity of native insect communities (Riddick et al., 2009).

Several studies have used plant secondary compounds, specifically sweet fennel essential oil (Digilio et al., 2008; Lacotte et al., 2023; Pavela, 2018; Soylu et al., 2006; Darwish 2021) for pest and fungal management on agricultural crops. They provide a natural and organic product that is readily available, affordable, biodegradable, and with minimal health risks for humans and animals (Digilio et al., 2008; Pavela, 2018). Therefore, understanding the role of plant secondary compounds on pathogens in natural enemies will help to ensure that beneficial insects remain effective biological control predators as an alternative to synthesized pesticides.

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