THE ECOLOGY OF APHID HYPERPARASITOIDS

by

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ABSTRACT

Aphids are economically important pests of protected horticulture crops such as sweet pepper (*Capsicum annum* Linnaeus 1753). Their control is partially achieved through augmentation biological control programmes based on supplemental releases of mass-reared primary parasitoid wasps. The efficacy of primary parasitoid wasps is, however, negatively impacted by naturally occurring hyperparasitoid wasps within the cropping environment. This project aimed to build upon existing knowledge of hyperparasitoid biology and ecology to aid the development of management tools that improve aphid IPM efficacy in UK protected sweet pepper crops. Experiments focussed on *Myzus persicae* Sulzer 1776, six commercially available primary parasitoid species: *Aphidius colemani* Viereck 1912*, A. ervi* Viereck 1912*, A. matricariae* Viereck 1912*, Aphelinus abdominalis* Dalman 1820*, Ephedrus cerasicola* Satry 1962 and *Praon volucre* Haliday 1833 and *Asaphes suspensus* Nees 1834*,* a hymenopteran hyperparasitoid common in United Kingdom sweet pepper cropping systems.

The development of these species was observed and recorded with *M. persicae* taking, a mean of, seven to eight days to reach reproductive adulthood from first instar nymphs. The three *Aphidius* species took a mean of 13 to 14 days to develop from the point of oviposition to adult emergence, *E. cerasicola* took just between 14 and 15 days a mean of, *P. volucre* took the longest time with a mean of 17.4 days. *Asaphes suspensus* took between 17 and 19 days to fully develop from oviposition to emerging adult which was not significantly affected by which of the six parasitoid species it used as its host. This shows that hyperparasitoid development is not affected by which primary parasitoid species is used by a grower for aphid control.

The apparent preference of primary parasitoids for specific aphid host developmental stage was investigated using choice and no-choice experiments offering individual parasitoid species *M. persicae* of different ages. This study validates previous studies that conclude *A. colemani* has higher parasitism levels in third instar *M. persicae* over all other life stages when given no choice between aphid developmental stage but also selected third instar aphids the most when given a choice of aphids at different developmental stages (Sampaio et al., 2008). Experiments carried out in Chapter Four investigated the impact of hyperparasitoid presence on a host plant has on primary parasitoid foraging behaviour. This experiment demonstrated that primary parasitoids parasitised fewer aphids when there had been four or eight hyperparasitoids previously present on the leaf, likely suggesting hyperparasitoids deposit a non-volatile semiochemical cue that the primary parasitoid detects and that might affect their oviposition success. This research has provided a foundation for hyperparasitoid research to develop the chemical ecology of hyperparasitoids and their use of semiochemicals in host selection. Further information on this could be used in developing hyperparasitoid monitoring and control techniques in the UK's protected sweet pepper crops.

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CHAPTER 1

LITERATURE REVIEW: ECOLOGY OF APHID HYPERPARASITOIDS

1.0 Introduction

1.1 Protected Sweet Pepper Crops in Western Europe

1.1.1 History and Economic Value of Sweet Pepper Production

Sweet pepper (*Capsicum annum* Linnaeus 1753), also known as bell pepper, originated in Central America and northern South America where dried fruits and seeds have been discovered in 9000-year-old burial sites (Laborde and Rendon-Poblete, 1989). It is thought to be one of the first crops to be domesticated in the Americas, around 12,000 years ago (Park et al., 2016). Dried peppers were first brought to Europe from the West Indies in 1493 by Christopher Columbus, and from there cultivation spread to Africa, Asia and North America (Andrews, 1995). In 2018, the biggest producers of sweet pepper, by economic value, were China (US$ 13.8 billion), Indonesia (US$ 5.2 billion), Republic of Korea (US$ 3.3 billion), Mexico (US$ 1.5 billion) and Spain (US $1.2 billion) (Food and Agriculture Organization of the United Nations, 2020). In 2018, the UK produced 20,640 tonnes worth £40 million (Food and Agriculture Organization of the United Nations, 2021).

1.1.2 Description and Production

Sweet peppers are botanically classified as berries and so typical convention is to refer to them as fruits. This convention shall be followed here. They are in the Solanaceae family of plants. This plant family also includes other economically important crops such as potatoes, tomatoes, aubergine and chilli peppers (Bussmann et al., 2020). Sweet pepper fruits are bell shaped with a glossy covering that varies in colour from red, orange or yellow to brown, black, white or purple (Nazzaro *et al.*, 2008).

Unlike most berries, sweet pepper seeds are surrounded by air in a hollow space (Quagliotti et al., 1981). For seed production, seeds are harvested from mature sweet pepper fruits after a few weeks of post-harvest ripening and can be stored for up to three years in cool, dry conditions (Vidigal et al., 2011). In Western countries, some protected sweet pepper production (i.e. in a glasshouse) incorporates seed priming as a technique to stimulate germination by treating seeds with moisture and cold temperature conditions between 1-7°C before sowing them (Siri et al., 2013). Typically, fewer seeds are required per hectare following seed priming due to increased germination rates. Seedlings are often transferred to pots once the first cotyledon (seed leaf inside the seed embryo) develops.

When grown under optimum temperature conditions (24 °C), it will take approximately 40 days from sowing for the plant to develop up to ten leaves (Albert, 2021). Growers of protected sweet pepper crops, growing the crop within a glasshouse, will import bees (Hymenoptera: Apidea) such as *Bombus terrestris* to increase crop yields by pollinating sweet pepper flowers, which will occur shortly after the flower opens and the anthers are exposed (Pereira et al., 2015). The pollen is picked up by the stigma of another sweet pepper flower, with pollinated flowers then developing into fruit. These fruits begin as a green pepper that takes approximately three to four weeks to grow and ripen, becoming the colour of a specific variety. The fruits are typically harvested only a month after pollination whilst the fruit is still green. These unripe fruits are left to fully mature off-plant so that the plant's energy can be spent on those fruits that still need to grow further. The fruits removed from the plant will also most likely ripen in time with those left on the plant which helps to minimise crop losses and maximise

profitability (Eggink *et al.*, 2012). Although sweet pepper is a tropical species of plant, it can be produced year-round in temperate regions when grown in protected environments such as glasshouses (Berke et al., 2003).

1.2 Aphid Pests of Sweet Pepper

1.2.1 Classification, Morphology and Feeding

Aphids are a large group of insects belonging to the order Hemiptera (Sternorrhyncha: Aphidoidea) that contains approximately 5,000 species worldwide, most of which inhabit temperate regions (Pilson, 1992). Members of this group are typically 1-10 mm long, soft bodied, pear-shaped and either winged (alate) or wingless (apterous) (Dixon and Thieme, 2007). Aphids are characterised by several diagnostic features, perhaps the key one being the pair of siphunculi (singular = siphunculus), otherwise referred to as cornicles, located dorsally on the fifth abdominal segment (Dixon and Thieme, 2007) (Fig. 1-1). These structures have an opening through which many species secrete an oily liquid to expel excess sugars and for use in response to perceived threats. This liquid may become smeared onto the mouthparts of predators, thereby immobilising them (Zabaras *et al.*, 1999; Pasteels, 2007). Part of this secretion is an alarm pheromone that is used to warn conspecifics of danger (Zabaras et al., 1999). The principle chemical component of the aphid alarm pheromone has been identified as (E)-β-farnesene that, when detected, evokes a behavioural responses from conspecifics (Francis et al., 2005). The specific behavioural responses depend on the aphid species but generally the detection of conspecific alarm pheromones will result in the aphid stopping feeding, moving away from the alarm pheromone source and often dropping from the plant (Pickett et al., 1992).

At the tip of the abdomen is the cauda, an appendage, much like a tail, that is usually conical shaped with setae, though in some species they are much more reduced and dome shaped (Gillette, 1927). Honeydew is a clear, syrup-like liquid excreted from the aphid's cauda and then flicked away by the hindlegs (Auclair, 1958; Cristofoletti et al., 2003) (Cristofoletti et al., 2003) (Fig. 1-1).

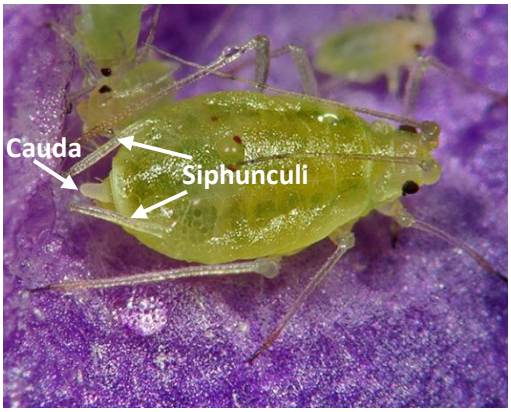


Figure 1-1: Apterous *Myzus persicae* Sulzer being 1.2 – 2.3 mm long, showing the rounded soft-body, the siphunculi and cauda (InfluentialPoints.com, 2015).

As Hemiptera, aphids have piercing mouthparts to penetrate the epidermis of their host plant and move intercellularly through plant tissue to reach the phloem. Once the phloem has been reached, the mouthparts pierce a phloem cell, which is under high pressure, causing the

phloem to move up the stylets allowing the aphid to feed on the sap (Will and Vilcinskas, 2015). These mouthparts are known as stylets, which consist of two exterior mandibular stylets that act to protect two inner maxillary stylets from damage (Uzest et al., 2010) (Fig. 1-2A). The stylets lie within a grooved rostrum (Forbes, 1977) (Fig. 1-2B). The rostrum is thought to aid stylet orientation when piercing the plant and to provide stabilisation from the surface of the plant as the rostrum does not enter the plant, preventing physical damage to the aphid. It is also thought to seal any damage caused to the plant surface after piercing (Morgan et al., 2013).

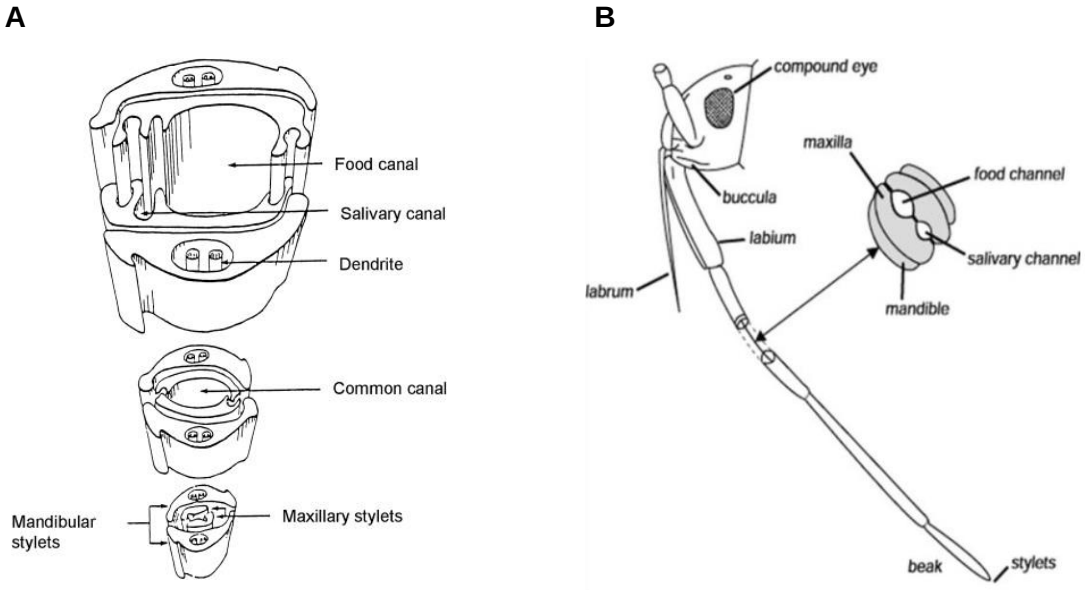


Figure 1-2: (A) Diagram of aphid stylet showing the food and salivary canals in the top panel; the common canal in the top and bottom panel at the distal extremity of the stylet (Uzest et al., 2010); (B) Diagram of an aphid's head showing the mouthparts made up of stylets encased in the labium which form the rostrum (Hamshou, 2012).

Aphids produce two kinds of saliva when feeding, (1) a watery saliva when penetrating plant cells and when ingesting the sap and (2) a more viscous gel-like saliva when the stylet is moving between the plant cells through the apoplast (the space outside of the plasma membranes where material such as water and nutrients can move more freely) where the saliva will harden to form a continuous sheath that protects the stylets from any damage whilst in the plant tissue (Sattelmacher, 2001; Will and Vilcinskas, 2015) (Fig. 1-3).

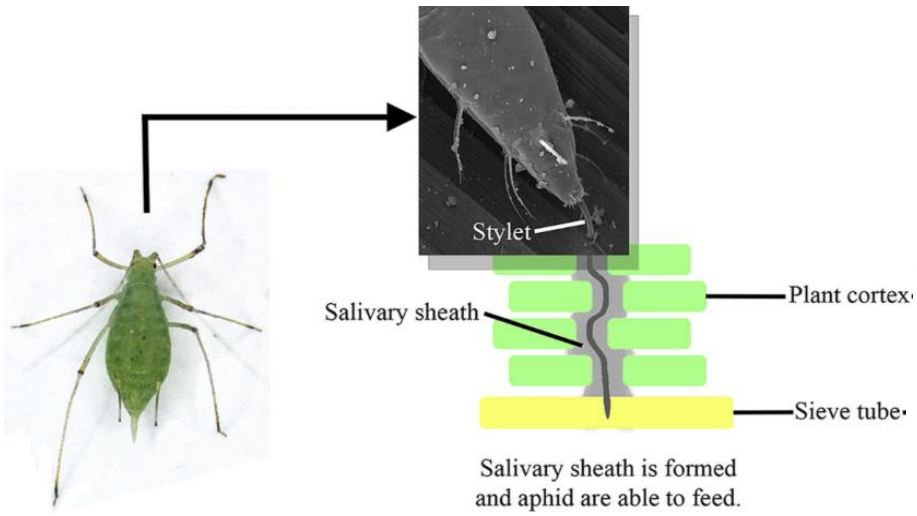


Figure 1-3: Aphid feeding showing the salivary sheath that will harden shortly after secretion to form a continuous sheath encasing the full length of the stylet whilst inside the plant (Will and Vilcinskas, 2015)

Plant phloem sap is predominantly consisting of highly concentrated sugar that creates a diffusion gradient drawing water into the phloem cells from the xylem (plant tissue that transports water and minerals from the roots to the stem and leaves), creating hydrostatic pressure (Patrick, 2012). This pressure would be fatal to an aphid as the high pressure would damage their digestive system if it weren't for the pharyngeal pump in the head of these insects, which can enlarge and decrease the size of the pharynx in the mouthparts, thereby controlling the quantity of sap moving up the stylet (Ponsen, 1991). There are also a series of valves that control the rate that the sap enters the aphid's digestive system (McLean and Kinsey, 1984). A second challenge faced by aphids feeding on phloem originates from the fact that sugar-rich sap would usually draw water out of an insect's own cells by osmosis, dehydrating the insect (Pompon et al., 2011). To prevent this from happening, aphids possess the enzyme sucrase-transglucosidase in their digestive system to adapt to the high-sugar content of the sap; the activity of this enzyme reduces water loss (Ashford *et al.*, 2000; Douglas, 2006). Though the sap has a high sugar content it is nutrient-poor (Douglas, 1998). As phytophagous insects, nitrogen is a major factor that limits the growth and development of aphids (Pandharikar et al., 2020). To acquire sufficient nitrogen from phloem sap, aphids ingest a large quantity of sugar; most of which is not assimilated and passes through the aphid to be excreted as honeydew (Auclair, 1958). Most aphids also have a symbiotic relationship with bacteria of the genus *Buchnera*, which aid in the production of essential amino acids (Douglas, 2006).

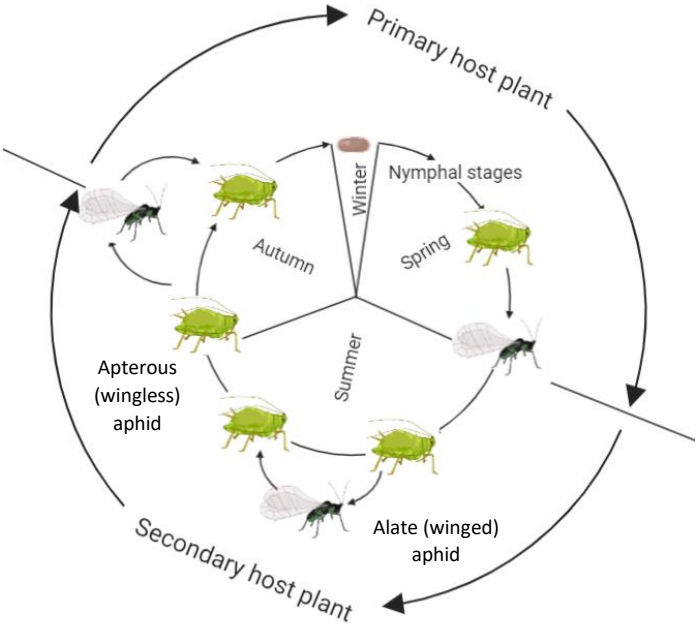
1.2.2 Aphid Ecology

Aphids are hemimetabolous and therefore undergo incomplete metamorphosis (Moran, 1992). Holometabolous metamorphosis is seen when an insect develops through morphologically distinct stages and there is a pupal stage between the larval and adult stages of the life cycle (Rolff *et al.,* 2019). During the pupal stage, the insect will undergo major tissue

and organ remodelling to transform the larvae into the final adult form. This does not occur in hemimetabolous insects such as aphids (Moran, 1992). Aphids give birth to viviparous (asexual) live young that largely look like the final adult form (Vilcinskas, 2016). They will moult, discarding their exoskeleton, as they grow. The growth stages of their development, separated by the discarded exoskeleton, are known as instars. There are typically four instars before the aphid reaches its adult form (Moran, 1992).

Two types of aphid life-cycle have been described: host-alternating (heteroecious) and non-host-alternating (autoecious) (van Emden and Harrington, 2017). Heteroecious aphids overwinter on one plant host, usually a woody plant, known as the primary host. In the spring/summer they migrate to a secondary host plant, usually a herbaceous plant, before migrating back to the original host plant in the autumn (Fig. 1-4A) (Moran, 1992). Approximately 90 % of aphid species are autoecious (sometimes referred to as monecious) and as such do not alternate between woody and herbaceous host plants, or at least colonise only closely-related plant species (Fig. 1-4B) (Moran, 1988).

A. Heteroecious life-cycle



B. An autoecious life-cycle

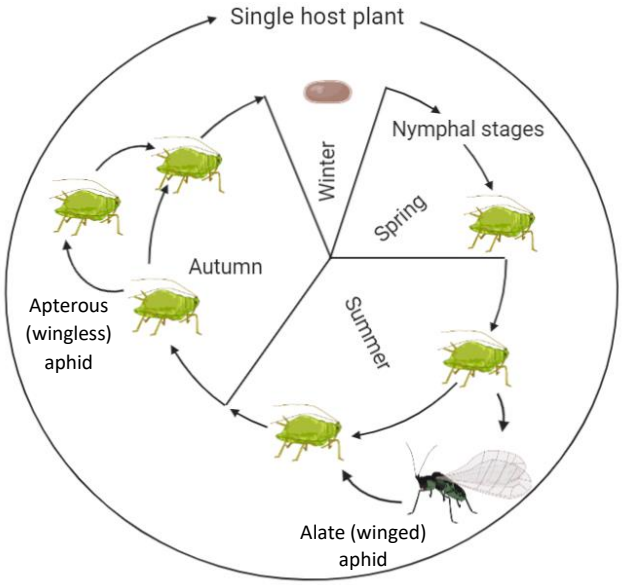


Figure 1-4: (A) Heteroecious aphid life cycle where the primary plant host is colonised in the winter, then the secondary plant host is colonised in the spring and summer (van Emden et al., 1969). (B) Autoecious aphid life cycle where the same or similar host plant species are colonised throughout the year. Winged (alate) forms occur in cases of overcrowding and disperse to a new host plant (Emden and Harrington, 2017). (Created by author with BioRender.com).

In holocyclic aphid species the life cycle will begin with fundatrices, or stem mothers, hatching in the spring from overwintering eggs. Fundatrices develop through their nymphal stages until they reach adulthood. As adults these aphids reproduce asexually through parthenogenesis meaning that the eggs do not require fertilization and will develop into female offspring (Suomalainen, 1950; van Emden and Harrington, 2017). Following fundatrices there are several generations produced parthenogenetically throughout the spring and summer (Sing and Sing, 2016). Sexual reproduction is known to rarely occur in *Myzus persicae* (Sulzer 1776) as day length begins to decrease in the autumn. The apterous viviparae of many holocyclic species such as *M. persicae* (Guillemaud *et al.,* 2003) and the potato aphid (*Macrosiphum euphorbiae* Thomas 1878) (Hurley *et al.,* 2014), may produce gynoparae (parthenogenetic viviparous aphids) and males on the secondary host plant which then migrate to the primary host plant. Having migrated to a suitable primary host, the gynoparae then give rise to a sexual generation consisting of oviparae (egg-laying females) which mate with the winged males (Dixon, 1975). This combination of both parthenogenic and sexual reproduction in holocyclic aphids can occur in both heteroecious and monoecious populations. Anholocyclic populations are those that only reproduce parthenogenically as they do not produce a sexual generation and so do not lay eggs (Pons et al., 1995). In response to overcrowding, alate individuals arise to allow for migration between hosts. This has not be observed in UK, however.

1.2.3 Aphid Damage to Sweet Pepper Crops

Sweet pepper is a high-value crop susceptible to aphid infestation; its most common aphid pest being *M. persicae* (Weintraub, 2007). This aphid species is autoecious (non-host alternating) and anholocyclic (reproduces parthenogenically only) in the UK, though it is heteroecious (host alternating) and holocyclic (can reproduce sexually or parthenogenetically) outside of the UK (Margaritopoulos et al., 2002). Aphid feeding on the crop as nymphs and as adults causes distortion, stunted growth, honeydew build-up, sooty mould development and white spots on the pepper fruit if feeding occurred on young fruit buds (Weintraub, 2007) (Fig 1-5). Many aphid species act as vectors; transmitting plant viruses (Shah *et al.*, 2015). *M. persicae,* for example, is an effective vector of yellow mottle mosaic virus in sweet pepper plants, which can devastate whole crops by stunting growth, mottling the leaves and causing smaller and lumpy fruit (Su, 1982). Aphids can appear in the very early stages of sweet pepper development and so may be present during transplanting (Chantal and Ramakers, 2008). Early infestation of the sweet pepper crop is of particular concern as sweet pepper is more susceptible to feeding damage as a seedling while older plants are better able to tolerate aphid infestations (Polack *et al.,* 2011).

The Agricultural Standards Unit, Trade and Timber Division of the United Nations Economic Commission for Europe outlines commercial standards to growers regarding how their sweet pepper fruit should look and taste ("UNECE standard on the marketing and commercial quality control of sweet peppers," 2009). Sooty mould on the fruit is unacceptable, along with any external and internal pest damage and the presence of any living, dead or exuviae of aphids. Should the crop develop to full maturity and be harvested, growers must spend considerable time and money cleaning any honeydew, mould or insects from the fruit. Yield losses along with the cost of controls and washing of produce post-harvest can cost an average sweet pepper grower more than £100,000 per hectare per season (Jacobson, 2010).

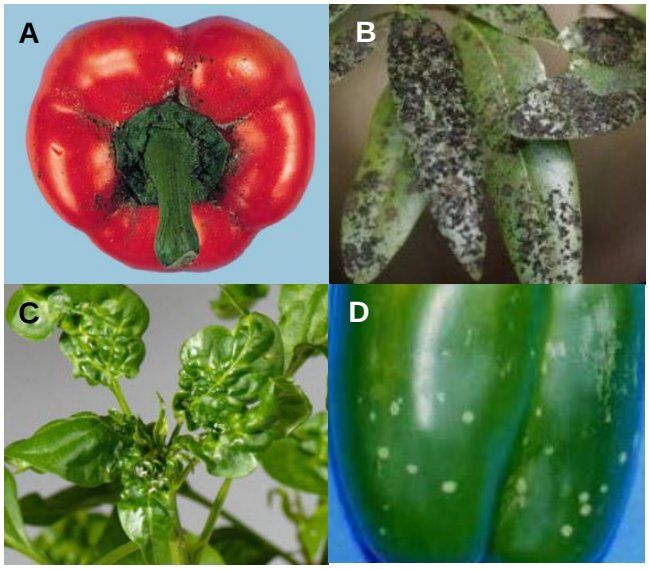


Figure 1-5: (A) sooty mould on a sweet pepper fruit developing on aphid honeydew (USDA Forest Service, 2012); (B) sooty mould on sweet pepper leaves developing on aphid honeydew (UNECE, 2009) (C) distorted plant growth as a result of aphid feeding (Alamy, 2017); (D) white spots on pepper fruit caused by direct feeding damage by aphids when the fruit was still a bud (USDA Forest Service, 2012).

1.3 Aphid Management in Sweet Pepper Crops

1.3.1 Integrated Pest Management

Integrated pest management (IPM) is a holistic strategy to pest management that is inclusive of all available control methods (Stenberg, 2017). The strategy is not employed with the aim of eliminating the pest but to manage their population(s) to keep them below economically damaging levels (Stenberg, 2017). It has been defined as 'a sustainable approach to managing pests by combining biological, cultural, physical and chemical tools in a way that minimises economic, health and environmental risks' (Schwartz and Peairs, 1999). Stenberg (2017) highlights a need for further research into IPM systems and their application as they argue that no strategy deemed as IPM that has matched all criteria to be seen considered holistic. Stenberg counters the given definition of IPM by referring to it as a 'systematic study of the compatibility and optimization of actions associated with at least two pest management elements', such elements are outlined below and in figure 1-6.

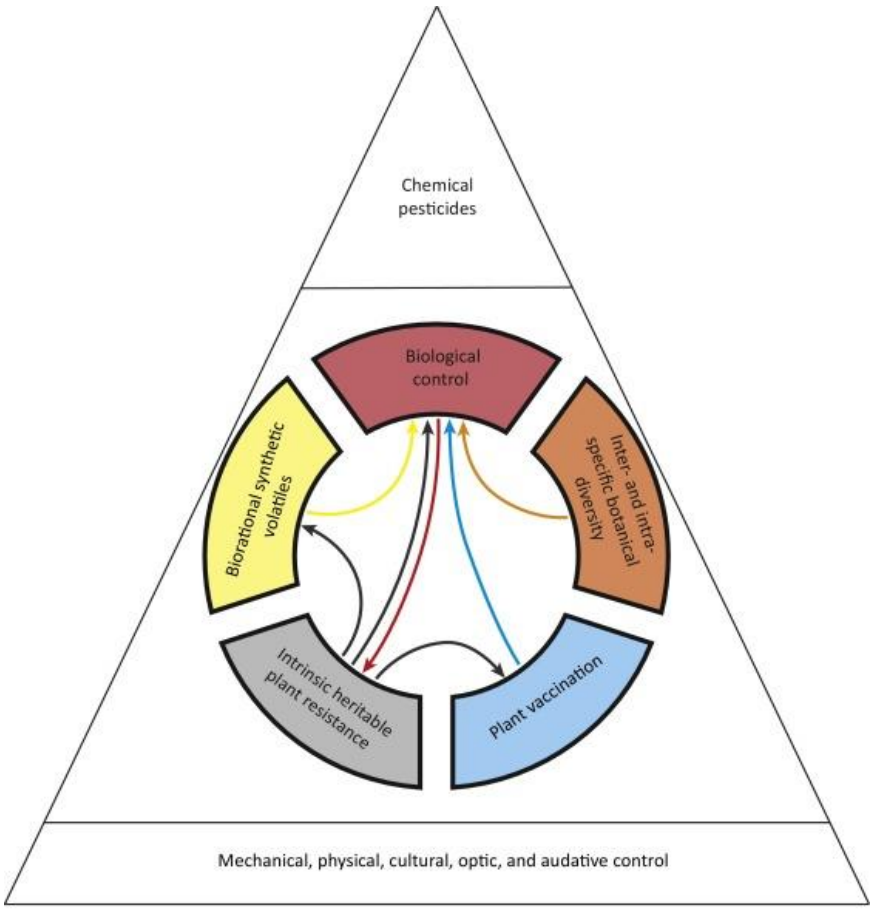


Figure 1-6: The Integrated Pest Management Pyramid used to display the key strategies used in IPM where the arrows demonstrate where one strategy can be used as a key component of another strategy (Stenberg, 2017).

Resistance has evolved in many wild plants as defence against herbivores and pathogens (Bennet and Walsgrove, 1994). The characteristics that some plants have evolved in defence of herbivores can be separated into direct and indirect defences (Stenberg, 2017). Direct defences include secondary metabolites, which include toxins and deterrents that

prevent organisms from feeding on the plant (Bennet and Walsgrove, 1994). There are also physical defences such as trichomes, which are sticky glandular crystals or hooks that may cover the surfaces of leaves and/or flowers (Levins, 1973) and wax layers that act as a feeding deterrents for many insects (Holmes and Keiller, 2002). Indirect defences relate to attracting species that act as natural enemies of the herbivores through release of specific odours and/or nectar rewards (Stenberg, 2017). Through the domestication of crops, many plant defences have been lost either as a result of them being actively selected against, as with direct defences such as trichomes and wax layers which are considered unpleasant textures for the consumer, or through a lack of consideration such as with indirect traits (Chen et al., 2015). Research has shown that these traits are present within wild crops (Tamiru *et al.,* 2011). Developments within IPM include research into finding these resistance traits and breeding them into crop cultivars (Ramírez-Carrasco et al., 2017).

Direct and indirect plant defences against herbivores, as outlined above, help the plant to protect itself when attacked and to prevent future attacks. 'Plant vaccination' is a term used to describe a process whereby a herbivore triggers these defences and this causes the plant to be more resistant to more threatening species (Kessler and Baldwin, 2004). 'Priming' crops is a technique used in agriculture whereby this vaccination process is carried out so that the crop will respond more readily and exhibit a level of resistance to detrimental pests and other biotic and abiotic stresses (Luna, 2016; X. Wang et al., 2017). For example, *Cis*-Jasmone is an important component of natural plant volatiles released as a defence in response to damage, from insect herbivory for example (Pareja et al., 2007). Spraying a solution of *Cis*-Jasmone onto crops has been shown to make the crop less attractive to *M. persicae* which shows great promise in using *cis*-Jasmone as an important component of integrated pest management (Ali et al., 2021).

Intra-specific botanical diversity refers to genotypic variation within the same plant species, known as different cultivars when referring to crops (Huel and Hucl, 1996). Inter-specific botanical diversity refers to different species of plants within the cropping system (Li et al., 2019). The lack of genetic variation in monocultures means that there is no variation in pest resistance and even where a single cultivar is selected for its pest resistance, its overall effectiveness is still less than that of a mixed culture (Andow, 1983). Cropping systems that exhibit intra-and/or inter-specific botanical diversity have a better chance of minimising pest problems than monocultures (Andow, 1983). Inter- and intra-specific biodiversity can be further improved by deliberately selecting plant species and cultivars that harbour traits that will attract beneficial species (e.g., predators or parasitoids of the pest) and those that will repel the pests (Pickett et al., 2014). Incorporating inter- and intra-specific diversity and deliberately manufacturing a push-pull strategy, whereby some form of stimuli is used to make the crop unattractive or unsuitable to the pest, *pushing* it away from crop, and *pulling* the pest to an attractive source such a pheromone trap, can greatly improve the effectiveness of IPM strategies by reducing the pest problem and, therefore, the need for synthetic insecticides for their control (Stenberg, 2017).

Biorational refers to products that are used to improve overall plant health, from pest management to stress management, with low environmental impact as they're either biological products such as sex pheromones or derivatives of such biological products (Valent BioSciences, 2013). A pheromone is a chemical released from an animal into the environment that affects the behaviour or physiology of conspecifics that detect it. By extension, a sex pheromone is a chemical released by a male or female that stimulates a behavioural reaction in the opposite sex of the same species to aid in them locating one another for the purpose of mating (Nakagawa *et al.*, 2005). Sex pheromones are amongst some of the more promising

biorational synthetic volatiles (Witzgall et al., 2010). These pheromones can be implemented within IPM systems as a way of luring the males of a pest species away from a crop and affecting their ability to find a female mate, thereby reducing reproduction of the pest within the cropping system (Lo, Walker and Suckling, 2015). Additionally, biorational volatiles that can be used in pest management within cropping systems include plant volatiles that affect the behaviour of pests (Kergunteuil et al., 2012). Current research is investigating the potential of using synthetic plant volatiles to devise a push-pull strategy within cropping systems (Hatano et al., 2008).

Synthetic insecticides used in conventional cropping systems are often incorporated into IPM strategies. Conventional cropping systems rely on the use of, usually broad spectrum, synthetic insecticides to control aphids and other invertebrate pests (Fray et al., 2015). Insecticides can be divided into two categories: systemic, where the insecticide is able to move through plants, typically through the phloem, providing residual or long-lasting activity, and contact, where the insecticide kills the pest on point of contact and typically has less residual activity (Sparks and Nauen, 2015).

Sulfoxaflor (e.g., Isoclast), a fourth-generation sulfoximine insecticide, is an example of a systemic insecticide used by UK growers (Siviter et al., 2018). Sulfoxaflor became available to growers in the UK in 2007 and is the most widely used insecticide in UK sweet pepper crops following the withdrawal of pymetrozine in the same year, an insecticide that targets sap feeding insects by disrupting their feeding behaviour (Ausborn et al., 2005; Harrewijn and Kayser, 1997; Rezk et al., 2019). Sulfoximine-based insecticides are, to some extent, replacing neonicotinoids such as imidacloprid, thiamethoxam and clothianidin, which were banned in the UK due to their non-target effects on pollinators (Carreck, 2017). However, sulfoxaflor is also highly toxic to pollinating insects and it is, therefore, recommended to apply sulfoxaflor to plants when pollinators are less likely to be active which can be easily done within protected cropping systems in the UK (Siviter et al., 2018). Studies suggest that sulfoxaflor has potential to be incorporated into IPM strategies should its application be carried out with consideration of pollinating insects and when used preventatively early in the season (Tran et al., 2016). Additionally, a group of insecticides commonly used in conventional sweet pepper cropping systems is the pyrethroids (Jardim *et al.*, 2018), which are synthetic forms of naturally occurring pyrethrins produced by chrysanthemum flowers as an anti-herbivore defence (Matsuo and Mori, 2012). This group of chemicals has a low level of toxicity to mammals and birds but are highly toxic to fish (Wouters and van den Bercken, 1978; Gibson *et al.,* 1982). Pyrethroids are contact acting insecticides that target the sodium channels in the central nervous system of the insect and quickly cause paralysis and death (Davies et al., 2008).

Biopesticides are crop protection tools derived from living micro-organisms or other natural products, such as bacteria, fungi, oomycetes, viruses and protozoa, to manage pests (Chandler et al., 2011). They are frequently used within IPM strategies and offer an alternative to synthetic insecticides that do not carry the same risk regarding toxicity to other animals, including beneficial insect species or in contaminating soil, other vegetation, and aquatic habitats. They typically being just as effective in controlling pests as synthetic insecticides (Mellanby, 1967; Pimentel et al., 2005, Barnett et al., 2007; Georghiou,). An example of a class of widely used biopesticides are fatty acids to target soft bodied insects like aphids (Siegler and Popenoe, 1925). FLiPPER is a product available in the UK formulated from unsaturated carboxylic fatty acids derived from olive oil, which interfere with vital cellular activity in the targeted organism (FLiPPER, no date). This product is used for pest control in many crops such as strawberries, cucumbers, and tomatoes, including pepper and chillies.

FLiPPER is marketed as a 'bee-friendly' product that is safe to beneficial insects this is of value to cropping systems such as protected sweet pepper which import bumblebees for pollination. Additionally, kaolin and mineral oils are used in organic farming (Karagounis *et al.*, 2006). Kaolin is a clay mineral used as a spray in sweet pepper crops that covers the surface of leaves and fruit acting as a barrier to insects whilst promoting photosynthesis (Jifon and Syvertsen, 2003). It has been shown to significantly reduce sun scorch damage to the pepper fruit but not to significantly increase sweet pepper yields (Ćosić *et al.*, 2015). In a study on *M. persicae* in peach orchards, kaolin was shown to be as effective as imidacloprid at reducing pest damage and increasing crop yield (Karagounis et al., 2006). However, it is likely that within sweet pepper crops, growers would face some of the same challenges in the use of these biopiosticides as they do when using synthetic insecticides, such as the target pests developing resistance to the product, an effect that has been observed in *M. persicae* and the melon and cotton aphid (*Aphis gossypi* Glover 1877). The likelihood of which increases the more frequently the insecticide is used (Elbert and Nauen, 2003). Additionally, the difficulty in getting consistent and thorough coverage of the spray throughout the entire crop which would be apparent in synthetic insecticides and biopesticides due to the dense leaf canopy and the fact that sweet pepper crops are grown in close rows (Yu et al., 2009). Therefore, it is imperative that additional strategies are in place within IPM programmes that effectively contribute to the control of the pest.

Biological control is the use of living organisms to control pest species by reducing their population through predation or parasitism or by outcompeting the pest for resources (Eilenberg et al., 2001). It is the oldest form of non-chemical pest control and is arguably the most researched element of IPM strategies (Stenberg, 2017). Biological control methods may be an effective pest control strategy with reduced damage to natural habitats and beneficial species (Baker *et al.,* 2020). It is traditionally considered to consist of classical biocontrol; intentionally introducing an organism to the environment where a pest has invaded to establish a population for long-term pest control, inundative biocontrol; the use of biopesticides and conservation strategies (Stenberg et al., 2021). Other strategies within biocontrol include the conservation of naturally occurring natural enemies, introducing a natural enemy with the goal of establishing a population, mass rearing and release of natural enemies on a seasonal basis and augmentation biological control whereby additional natural enemies are released in cropping systems where there is already a population present but greater numbers are required for effective pest control (Hajek and Eilenberg, 2018). A core component of many IPM strategies against aphid pests in protected sweet pepper cropping systems is biological control (Powell and Jutsum, 1993). Most commonly, augmentation biological control is used whereby mass-reared natural enemies, such as predators and parasitoids, are released into the cropping system to supplement naturally occurring populations (Collier and van Steenwyk, 2004).

1.4 Primary Aphid Parasitoids

1.4.1 Ecology of Hymenopteran Parasitoids

There are approximately 68,000 species of parasitoid wasps described worldwide (Fernandez-Grandon, 2012), over 400 of which target aphids (Boivin et al., 2011). Most adult hymenopteran parasitoids feed on floral nectar or aphid honeydew to supplement their diet with carbohydrates (Charles and Paine, 2016) and are solitary endoparasitoids that lay their eggs inside their host as either koinobionts (the host may continue to move and eat beyond parasitism) or idiobionts (paralyzing the host at the point of parasitism) with the emerging larva then developing within their host before pupating and killing their host (Kavallieratos et al.,

2004). In *Aphidius* species this results in mummification, where the aphid becomes a bronze colour, rounder and rigid (Longley, 1999). It takes approximately one week to reach the mummification stage following oviposition, although the length of time is temperature and parasitoid species dependent (Zamani et al., 2007). Approximately one week after mummification in *Aphidius* species the wasp emerges from the pupa as an adult, chewing its way out of the mummified aphid (Boivin et al., 2011) (Fig. 1-7). Emergence leaves characteristic holes on the mummified host.

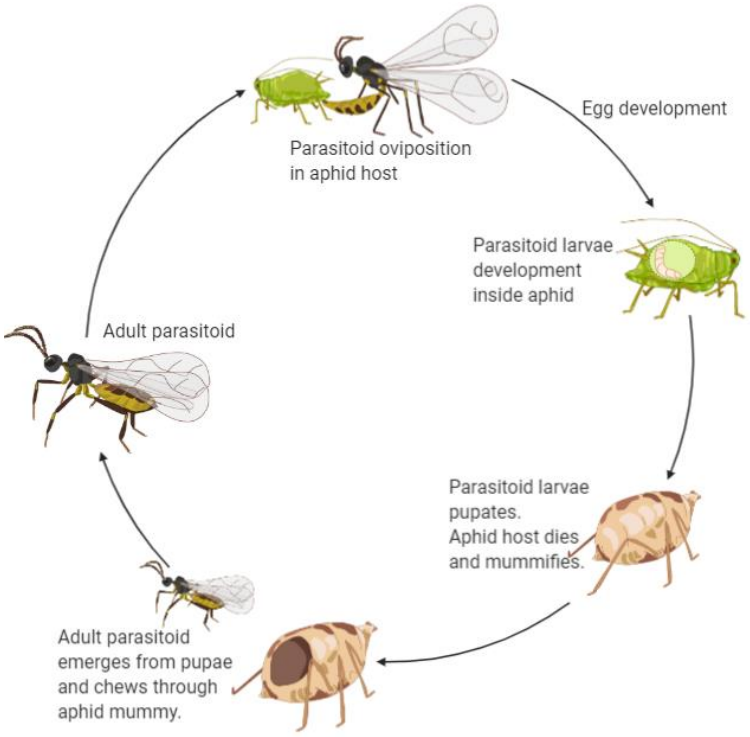


Figure 1-7: Life cycle of a primary parasitoid using an *Aphidius* species as host (after Fernandez-Grandon, 2012)

Parasitoids can either be described as a koinobiont or idiobiont. Koinobiont parasitoids do not prevent the host from developing once they have laid their eggs whereas idiobiont parasitoids prevent further development of the host by paralysing the host during oviposition (Otto and Mackauer, 1998). Some koinobiont parasitoids induce a transient paralysis in the host lasting no more than a few minutes (Desneux et al., 2009). Ectoparasitoids, those that lay their eggs on the outside of their host, are typically idiobiont species and it is thought that by preventing host activity the risk of the developing parasitoid being harmed is reduced (Otto and Mackauer, 1998). Koinobionts are usually endoparasitoids as their developing offspring are exposed to fewer risks by being protected within the host (Colinet *et al*., 2005) (Fig. 1-8).

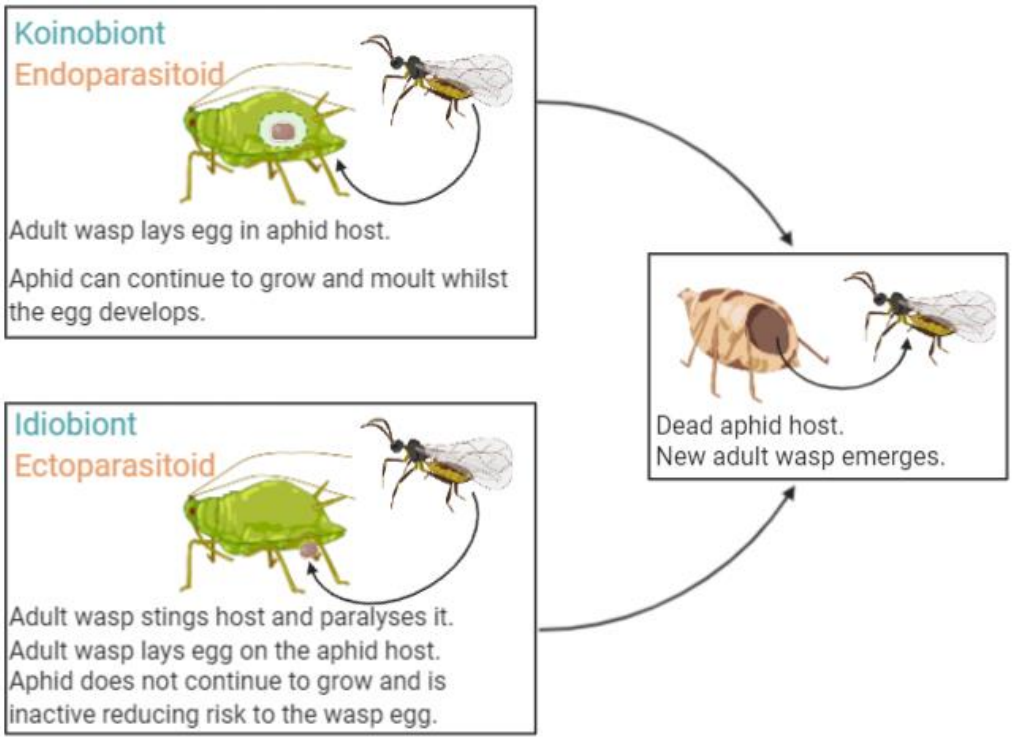


Figure 1-8: Koinobiont and idiobiont parasitoid strategies: ectoparasitoids usually being idiobiont to reduce risks to the developing wasps and endoparasitoids being koinobiont as the developing wasps are protected by the aphid.

1.4.2 Commercially Available Parasitoids

There are six hymenopteran parasitoids commercially available to growers for aphid control: *Aphidius colemani* Viereck*, A. ervi* Haliday*, A. matricariae* Haliday*, Aphelinus abdominalis* Dalman*, Epherdus cerasicola* Haliday and *Praon volucre* Haliday (Table 1-1). Parasitoid wasps can be used to control different pest aphid species, e.g., *Aphidius colemani* can successfully parasitise 40 species alone (Sampaio *et al.* 2013). This parasitoid wasp species is commonly used throughout Europe within ornamental and greenhouse vegetable crops (Vásquez *et al.*, 2006; Boivin *et al.*, 2011), being able to parasitise many aphid species but particularly effective against *M. persicae* and *Aphis gossypii* (Zamani et al., 2007). Typical practice is to release the parasitoids on a weekly basis early in the year as a preventative approach to aphid control with at least 0.15 individuals released per m^2(Goh *et al.*, 2001). Many growers will set up yellow sticky traps to monitor aphid populations and once they've been detected the number of parasitoids released is increased to 0.5-1 m^2/week for at least another three weeks (Goh *et al.*, 2001). Weekly releases of *A. colemani*, 0.15 – 2 individuals per m^2 means that through the sweet pepper growing season of nine to ten months, 30 releases of *A. colemani* can be made in one year (Goh *et al.*, 2001). Globally, the horticultural industry purchases 0.8-60 million *A. colemani* per year (Vasquez et al., 2006).

Table 1-1: Commercially available Hymenopteran parasitoids and the pests that they help to control (Sampaio *et al*., 2008).

Parasitoid species	Target pests	Retail packaging
Aphidius colemani	Peach-potato aphid (*Myzus persicae*) Cotton aphid (*Aphis gossypii*) Foxglove aphid (*Aulacorthum solani*) pea aphid (*Acyrthosiphon pisum*) Potato aphid (*Macrosiphum euphorbiae*)	Single species and as a mix with *Aphidius ervi, Ephedrus cerasicola and Aphelinus abdominalis*
Aphidius ervi	Peach-potato aphid (*Myzus persicae*) Foxglove aphid (*Aulacorthum solani*) Potato aphid (*Macrosiphum euphorbiae*) Mealy cabbage aphid (*Brevicoryne brassicae*)	Single species and as a mix with *Aphidius ervi, Aphelinus abdominalis and Ephedrus cerasicola*
Praon volucre	Potato aphid (*Macrosiphum euphorbiae*) Peach-potato aphid (*Myzus persicae*)	As a mix with *Aphidius ervi, Aphidius colemani, Aphidius matricariae* and *Aphelinus abdominalis.*
Aphidius matricariae	Peach-potato aphid (*Myzus persicae*) Cotton aphid (*Aphis gossypii*)	Single species and as a mix with *Aphidius ervi, Praon volucre, Aphidius colemani, Ephedrus cerasicola* and *Aphelinus abdominalis.*
Ephedrus cerasicola	Foxglove aphid (*Aulacorthum solani*) Peach-potato aphid (*Myzus persicae*)	Single species and as a mix with *Aphidius ervi, Aphidius colemani, Praon volucre, Aphidius matricariae* and *Aphelinus abdominalis.*
Aphelinus abdominalis	Foxglove aphid (*Aulacorthum solani*) Peach potato aphid (*Macrosiphum euphorbiae*) Peach-potato aphid (*Myzus persicae*)	Single species and as a mix with *Aphidius ervi, Aphidius colemani, Aphidius matricariae, Ephedrus cerasicola* and *Praon volucre.*

1.4.3 *Aphidius* spp.

Aphidius is a genus belonging to the Aphidiinae subfamily of the Braconoidae family; the second largest Hymenopteran family with 17,000 described species (Lins et al., 2013). The Aphidiinae subfamily includes koinobiont endoparasitoids that use aphids as their hosts (Belshaw and Quicke, 1997). Three of the six commercially available parasitic wasps for biological control belong to this genus.

1.4.3.1 *Aphidius colemani*

Adult *Aphidius colemani* are strong flyers, approximately 2mm long, black with yellow legs and long antennae with 15-16 segments (Fig. 1-9). This species has a petiole (a narrow waist between the thorax and abdomen), as is characteristic of Apocrita, and females have an abdomen that comes to a point that can be bent underneath the thorax when laying eggs inside the aphid (Fig. 1-10) (Stary, 1975). *Aphidius colemani* are most used against *M. persicae* infestations as well as *Aphis gossypii*) and the foxglove aphid (*Aulacorthum solani*).

Figure 1-9: Female *Aphidius colemani* showing the long antennae, yellow legs and petiole (Plant Protection, 2005).

Figure 1-10: Female *Aphidius colemani* ovipositing eggs inside an aphid host, curling the abdomen under the body (Green Methods, 2019).

Adult *Aphidius colemani* females live for approximately two weeks following emergence and will lay one egg inside each aphid host but are capable of laying several hundred eggs, with most eggs being laid within the first couple of days following emergence (Ode *et al.*, 2005). The mummified aphid will be bronze and once the pro-ovigenic (carrying matured eggs ready for oviposition) adult emerges from the pupa it will chew its way out of the mummy, leaving a small semi-circle shaped incision in the mummy exoskeleton (Fig. 1-11).

Figure 1-11: Exoskeleton of mummified *Myzus persicae* showing semi-circle shaped incision where the adult *Aphidius colemani* has chewed its way out (Author's own).

1.4.3.2 *Aphidius ervi*

Aphidius ervi naturally occur in most of Europe but it has been introduced to countries such as the United States of America, Canada, Australia, New Zealand and Argentina for its use as a biological control agent in protected cropping environments (Cameron *et al.*, 1984; McBrien and Mackauer, 1991). Adult size depends on the aphid host species it developed in, for instance when parasitising potato aphid (*Macrosiphum euphorbiae*) the emerging adults can be approximately 4 mm long (Sequeira and Mackauer, 1993). They have long dark wings and a black slender body with gold markings, brown legs and long antennae of 18-20 segments (Fig. 1-12) (Pennacchio and Tremblay, 1986) . Like *A. colemani,* the female will bend her abdomen underneath the thorax during oviposition (Battaglia et al., 2000). The aphid host will live and continue to feed once the egg has been laid as *A. ervi* is koinobiont, but once the egg hatches, larvae will feed on the aphid from the inside. The mummified aphid will be bronze and once the adult emerges from the pupa it will chew its way out of the mummy, leaving a small hole (Colinet *et al.*, 2005)(Fig 1-13). The development time varies depending on the average temperature; for example it is 12 days at 25 °C and 19 days at 18 °C. Each adult female may parasitise over 300 aphids within their two to three week lifespan (He *et al.*, 2004).

Figure 1-12: Adult *Aphidius ervi* showing long antennae, brown legs, gold markings on slender black body and petiole (Wikispecies, 2015).

Figure 1-13: Mummified pea aphid (*Acyrthosiphon pisum*) with a hole in the abdomen where an adult *Aphidius ervi* emerged (Martinez, 2011).

1.4.3.3 *Aphidius matricariae*

Aphidius matricariae adults are black and slender wasp around 2mm long. It has brown legs and long antennae about 2-3mm long (ANATISBioprotection, 2009) (Fig 1-14). Like the aforementioned *Aphidius* species, *A. matricariae* is koinobiont and the female bends her abdomen underneath the thorax during oviposition (J *et al*., 2011). The mummified aphid will

be bronze and the emerging adult will chew its way out of the mummy, leaving a small hole (J *et al*., 2011) (Fig. 1-15).

Figure 1-14: Female *Aphidius matricariae* adult showing dark body and brown legs, tucking her abdomen underneath her thorax to oviposit in an aphid host (*Matricariae-System* | *Biobest*).

Figure 1-15: A bronze and rounded *Aphidius matricariae* aphid mummy, typical of *Aphidius* species (ANATISBioprotection, 2009).

1.4.3.4 *Aphelinus abdominalis*

Aphelinus is a genus in the Aphelinidae family of approximately 1160 species in 35 genera of parasitic wasps (Japoshvili and Hansen, 2014). *Aphelinus abdominalis* is a species used commercially by growers to control aphid infestations. They are known to be primarily target larger aphid species, much like *Aphidius colemani*, such as the potato aphid as a result of its size as an adult, approximately 3 mm long (Japoshvili and Hansen, 2014). The adults are black with a yellow-brown abdomen and, unlike the aforementioned parasitoid species, they have relatively short antenna with 3-6 segments and legs (Biological Services, Australia) (Fig. 1-16). They also differ from *Aphidius* species in their oviposition technique where the female will keep her abdomen straight and extended distally to sting the aphid whilst facing away from it (Fig. 1-17). Adult females will begin oviposition approximately 3 days after emergence and can lay 5-10 eggs a day for several weeks (Wahab, 1985). The eggs hatch 2-3 days after oviposition and then pupate approximately another 7 days later, mummifying the aphid into a black mummy opposed to the bronze ones commonly seen in the other commercial parasitoids (Koppert Biological Systems) (Fig. 1-18). In addition to targeting a wide range of hosts and having a long lifespan and oviposition period, they are also predators of other non-host aphid species so are all-round effective biological control for growers (Honek et al., 1998).

Figure 1-16: Adult *Aphelinus abdominalis* showing a yellow abdomen and dark thorax and head and short antenna (Biological Services, 2015).

Figure 1-17. Female *Aphelinus abdominalis oviposition* by extending abdomen backwards as she faces away from the aphid (Biological Services, 2015)

Figure 1-18. Black aphid mummies parasitised by *Aphelinus abdominalis* (Biological Services, 2015).

1.4.4 *Praon volucre*

The *Praon* genus belongs to the Aphidiinae subfamily within the Braconidae family. *Praon volucre* is a koinobiont endoparasitoid of *Macrosiphum euphorbiae* and many other aphid species including *M. persicae* of which it is an effective control for in sweet pepper crops (Tazerouni et al., 2019). The adults have a dark head and thorax with a slightly more bronze coloured abdomen and legs and long antennae. Like *Aphidius* species, *P. volucre* females bend their abdomen beneath their body when laying eggs in their aphid host (Encyclop'Aphid, 2010) (Fig. 1-19).

Figure 1-19: *Praon volucre* female bending her abdomen below her body during oviposition in an aphid host and showing dark head and thorax, long antennae and bronze coloured abdomen and legs.

Praon volucre larvae secure the mummy to the leaf with web-like substance and the final instar of the wasp cuts itself out of the bottom of the aphid to spin its cocoon underneath the aphid (Beirne, 1942) (Fig. 1-20).

Figure 1-20: Aphid mummy parasitised by *Praon volucre* showing the cocoon that the parasitoid larva formed, attaching the aphid to the leaf.

1.4.5 *Ephedrus cerasicola*

Ephedrus cerasicola is a dark European parasitoid with yellow legs approximately 2mm long (Capinera, 2001) (Fig. 1-21). Like *Aphidius* species, *E. cerasicola* also bends its abdomen below itself to oviposit in its host (Fig 1-21).

Figure 1-21: *Ephedrus cerasicola* adult female laying an egg in an aphid host by bending her abdomen underneath her body.

1.4.6. Chemical Ecology of Primary Parasitoids

It is known that chemical cues play an intrinsic role in host location by parasitoids and that they can differentiate between aphid infested plants and uninfested plants (Pareja et al., 2007). The volatile profile of plants changes as a result of herbivory (Büchel et al., 2011), which attracts natural enemies of the pest feeding on the plant (Dudareva et al., 2006). Aphid parasitoids have been shown to positively respond to these volatiles when locating aphid hosts (Hatano et al., 2008). For example, *Aphidius ervi* has been shown to make more orientated flight toward herbivore induced plant volatiles (HIPVs) emitted due to aphid infestation, whereas non-herbivore induced volatiles emitted by the plant increase foraging time and lead to reduced fitness (Guerrieri et al., 1993).

1.5 Aphid Hyperparasitoids

1.5.1 Ecology of Aphid Hyperparasitoids

Hyperparasitoids, otherwise known as secondary parasitoids, are species that lay their eggs inside or on another species, which is itself a parasitoid (Sullivan, 1987). Hyperparasitism is present in some species of Diptera and Coleoptera and in 17 families of Hymenoptera (Sullivan, 2009). There are many genera of hymenopteran hyperparasitoids within three superfamilies that attack hymenopteran primary parasitoids: Chalcidoidea (Pteromalidae), Cynioidea (Alloxystidae) and Ceraphronoidea (Megaspilidae) (Sullivan, 2009). Some hyperparasitoid wasps are facultative, such as *Tetrastichus hawardi* (Kfir *et al.,* 2008), and can develop as either primary parasitoids or hyperparasitoids (Langellotto et al., 2006). Obligate hyperparasitoids, such as *Asaphes suspensus* Nees 1834 and *Dendrocerus aphidium* Kieffer 1907, can only develop through the immature stages of their life cycle in or on a parasitoid (Brodeur, 2000).

Much like primary parasitoids, aphid hyperparasitoid species will either lay their eggs in (endo-) or on (ecto-) their parasitoid host (Brodeur, 2000). They can also be generally be divided into two groups based on their feeding behaviour: (1) endophagous- consuming their host from the inside, seen in endohyperparasitoid species or (2) ectophagous- consuming their host from the outside, seen in ectohyperparasitoid species (Sullivan, 1987). *Asaphes suspensus* and *Dendrocerus aphidium* are ectophagous species that lay their eggs on the primary parasitoid once the aphid is already dead and mummified, leading to ovipositing females to make a hole in the rigid exoskeleton of the mummified aphid (Buitenhuis et al., 2005). Once the hyperparasitoid egg hatches, the larvae will feed on the larvae of the primary parasitoid from the outside whilst inside the aphid mummy (Singh and Singh, 2016). Like primary parasitoids, hyperparasitoids may be described as having either a koinobiont or idiobiont life cycle. *Dendrocerus* spp. and *Asaphes* spp. are ectoparasitic idiobionts; eggs are

laid on the host and the hosts are paralysed to prevent movement and further growth. Larvae of these two species of hyperparasitoid usually hatch from the egg once the aphid mummy has been formed by the primary parasitoid larvae (Harvey et al., 2012). Koinobiont hyperparasitoids, which do not paralyse their host or prevent its growth, are also typically endophagous and includes species of the family Alloxystinae (Buitenhuis, 2004).

1.5.2 Aphid Hyperparasitoids as Disruptors of Biological Control Programmes

The efficacy of parasitoid wasps as biological control agents is threatened by the presence of hyperparasitoids (Gómez-Marco et al., 2015). In a 2015 review, 16 representatives involved in sweet pepper production were interviewed and all were familiar enough with hyperparasitism to recognise its presence and understood it to be a threat to sweet pepper crops where there is reliance on parasitoids for aphid control (Fray et al., 2015). Typical practice once hyperparasitism is detected within a sweet pepper crop is to cease releases of aphid parasitoid and instead to switch to an insecticide to kill both the hyperparasitoids and aphids (Fray et al., 2015). Growers, however, recognise that this approach also results in the primary aphid parasitoids being killed. Following this insecticide application most growers release the aphid midge *Aphidoletes aphidimyza* Rondani for aphid control instead of returning to releasing aphid parasitoids. This results in more money and time spent on aphid control (Cusumano et al., 2020).

Multiple hyperparasitoid species can be present in the same cropping system at any one time (Lohaus *et al.*, 2013). *Asaphes* spp. and *Dendrocerus* spp., for example, are often found together within sweet pepper crops (de Boer et al., 2019). Co-existence may be explained by a range of factors: *Dendrocerus* may be successful earlier in the growing season as they can feed on honeydew whereas *Asaphes* thrives later in the season due to its long lifespan and extended reproductive period. Both genera will successfully develop in mummies at all stages of the mummification process (de Boer et al., 2019). For example, *Dendrocerus* is capable of successfully reproducing in recently mummified aphids where the aphid exoskeleton is still soft right up to the final stages of parasitism stages where the parasitoid is almost ready to emerge (de Boer *et al*., 2019). Species from both genera are known to survive in empty greenhouses over colder seasons so long as they have access to water and a source of sugar (de Boer, 2019). *Asaphes* spp. are the main hyperparasitoid species disrupting the biological control programmes used in greenhouse grown crops (Acheompong *et al*., 2012) (Cusumano et al., 2020).

1.5.3 Chemical Ecology of Aphid Hyperparasitoids

Recent studies have attempted to identify chemical cues used by hyperparasitoids in host selection as a first step in developing tools with which to manage hyperparasitoids (Zhu et al., 2011; Aartsma et al., 2019; Cusumano et al., 2020). The most frequently suggested approach to their control is to develop a push-pull strategy; pushing the hyperparasitoid from their parasitoid host and pulling them into a chemically-baited trap (Cusumano et al., 2020). To do this, however, understanding is needed regarding the semiochemicals used by hyperparasitoids in locating parasitised hosts. Evidence has shown that hyperparasitoids use aphid honeydew to locate the parasitoid host (Buitenhuis et al., 2004). Hyperparasitoid species that target caterpillar parasitoids have been shown to respond to HIPVs following caterpillar feeding (Poelman et al., 2011). Similarly, aphid hyperparasitoids are thought to exploit information from host plants and aphid hosts from a distance (Wahab, 1985). Ectohyperparasitoids are less host specific than endohyperparasitoids and are recorded to search randomly for their hosts (Schooler *et al.*, 2011). Some studies have shown that

ectoparasitoids do respond positively to cues when locating hosts (Strand and Godfray, 1989). For example, *Asaphes suspensus* has been recorded to positively respond to volatiles collected from mummified *Aulacorthum solani* aphids (Acheampong *et al.*, 2012). Studies such as this demonstrate that volatiles from parasitised aphids are used by hyperparasitoids, ecto- and endo-, in foraging. They, therefore, provide fundamental information to advance research into developing semiochemical baited traps as part of a push-pull strategy in controlling hyperparasitoid pests in cropping systems.

1.6 Thesis Aims and Objectives

1.6.1 Knowledge and Research Gaps

The fourth trophic level of hyperparasitism has been understudied, resulting in fundamental gaps in the understanding of their biology and ecology. A key area where knowledge is lacking can be found in the role of semiochemicals for host location and whether hyperparasitoids have a preference in the stage of parasitism in the hosts and if so, how they detect this stage within their environment (Aartsma *et al.*, 2019). Hyperparasitism is observed in certain families within three insect orders, Diptera, Coleoptera and, most importantly to growers, Hymenoptera (Sullivan, 2009). This can severely disrupt biological control programmes employed to control pest numbers, such as aphids (Araj et al., 2008). There remains a need for research on hymenopteran hyperparasitoids, particularly those species affecting aphid primary parasitoids, to improve understanding of the ecology of these species, and how they interact with the six species of parasitoids reared and sold by biological control companies, to develop practical solutions to reduce their populations within cropping systems. This work is required if biological control programmes are to be effective. Growers are under great pressure to produce high-quality products that meet commercial standards with a yield that supports their business and livelihood whilst simultaneously eliminating their use of synthetic chemical insecticides (Dassonville et al., 2012). Therefore, there is a great need for an effective control of hyperparasitoid pests that does not affect beneficial parasitoid species and reduces the need for additional insecticide applications (Fray et al., 2015).

1.6.2 Research Aims

This project aims to build upon the limited existing knowledge of hyperparasitoid biology and ecology to investigate the foraging behaviour of commercial parasitoids and how hyperparasitoid presence impacts it. This information will help develop management tools that improve aphid biological control efficacy in UK protected sweet pepper crops. The project aims to improve knowledge of hyperparasitoid biology to enable development of semiochemical based controls of these pests to be developed.

The project will conduct the following:

- Ecological studies on the life cycle of *M. persicae,* the 6 primary parasitoid species and the hyperparasitoid *Asaphes suspensus.*
- An age-preference study to investigate whether the primary parasitoid *Aphidius colemani* has a preference in the developmental stage of the aphid host. This will be investigated with two studies; a no-choice experiment where the parasitoid is presented with an individual aphid of pre-determined developmental stage and a choice experiment where the parasitoid is presented with a single aphid at each developmental stage at the same time to see which one it selects as a host. It is hypothesised that in both experiments the parasitoid would show a preference for the intermediate developmental stages (third and fourth instar).

- Studies on the presence of semiochemical trails following hyperparasitoid presence amongst the host plant and whether it effects primary parasitoid host selection. This was studied via the following six experiments:
    1. Effect of Adult Hyperparasitoid Trails on Primary Parasitoid Oviposition Success
    2. Effect of Hyperparasitised Mummy Exposure on Primary Parasitoid Oviposition Success
    3. Effect of Removing Adult Hyperparasitoid Cues on Primary Parasitoids Oviposition Success
    4. Effect of Removing Hyperparasitoid Mummy Cues on Primary Parasitoids Oviposition Success
    5. Removal and Re-Application of Anti-Oviposition Cues Associated with Adult Hyperparasitoid Exposure
    6. Removal and Re-Application of Anti-Oviposition Cues Associated with Hyperparasitoid Mummy Exposure

  It is hypothesised that hyperparasitoids deposit anti-oviposition cues on the host plant and that primary parasitoids will avoid ovipositing in aphids in these areas when left by the hyperparasitoid directly and when removed and reapplied to the leaves manually. It is also hypothesised that washing the leaves of the anti-oviposition cues will result in the primary parasitoids not being deterred from ovipositing in aphids on the washed leaves.

This information will help develop management tools that improve aphid biological control efficacy in UK protected sweet pepper crops. It is based on the premise that with this information will take steps forward into further research into the chemical ecology of aphid hyperparasitoid foraging behaviour. Taking these steps forward in hyperparasitoid knowledge will help work towards developing more efficient monitoring and trapping approaches such as the push-pull strategy discussed above. The project focuses on sweet pepper as the host crop and *M. persicae* as the aphid host. Initial studies on the development of the six commercial parasitoid species, *Aphidius colemani, A. ervi, A. matricariae, Aphelinus abdominalis, Epherdus cerasicola* and *Praon volucre,* will be undertaken with further study into the effects these different species may have on hyperparasitoid parasitism and development when used as hosts. The study will use *Asaphes suspensus* as the hyperparasitoid pest most commonly seen in sweet pepper cropping systems (de Boer et al., 2019).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Overview

This chapter provides a general outline of the materials and methods frequently used throughout the entire body of work presented. Materials and methods pertaining to individual experiments are described in their entirety within the relevant experimental chapters.

2.2 Plant Propagation

Sweet pepper seeds (*Capsicum annum,* var. 'California Wonderer') were sown approximately 5 mm deep into potting compost (John Innes N^{o}.2, J. Arthur Bower's, Dungannon, Northern Ireland) in a propagator tray and germinated in a controlled environment cabinet (Model SL2/RH-SL3/RH, LEEC Ltd, Nottingham, UK) at 30 ± 3 °C. Once germinated, the seed tray was transferred to an insect proof mesh cage (BugDorm: 60 x 60 x 60 cm, MegaView Science Co. Ltd, Taichung, Taiwan) housed within a glasshouse. The glasshouse environment was set to a 16:8 photoperiod (light:dark), 85 % RH and 15 °C during light hours and 5 °C dark hours. Once the plants reached BBCH (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) growth stage 1; having one set of true leaves, they were transplanted to 9 cm plastic pots and placed into a clean insect proof mesh cage (BugDorm: 60 x 60 x 60 cm). Plants were watered regularly by filling the tray inside of the cage with 5 mm of tap water whenever the soil surface was dry. The plants were left to grow to BBCH growth stage 2, with two true leaves, before use in the studies as it made it easier to locate the insects upon the plant for observation.

2.3 Insect Rearing

2.3.1 Aphids

Peach potato aphids (*Myzus persicae* Sulzer 1776) (Hemiptera: Aphididae) were maintained on sweet pepper plants (*Capsicum annum* var. California Wonderer) in insect proof mesh cages (BugDorm: 60 x 60 x 60 cm) housed within controlled environment rooms (Fitotron, Weiss Technik UK limited, Loughborough, UK) set to 20 ± 1 °C, 60 ± 5 % RH and a 18:6 photoperiod (light:dark). Between 6 and 12 clean pepper plants were added weekly to the culture to replace infested pepper plants removed from the aphid culture to maintain the primary parasitoid cultures. Plants were regularly watered by filling the tray inside of the cage with 5 mm of tap water whenever the soil surface was dry. Aphid cultures were inspected at least three times per week to assess whether the plants needed to be watered or additional clean pepper plants should be added in response to population over-crowding.

2.3.2 Primary Parasitoids

Six primary parasitoid species were reared for use in this project; *Aphidius colemani* Viereck*, A. ervi* Haliday*, A. matricariae* Haliday*, Aphelinus abdominalis* Dalman*, Epherdus cerasicola* Haliday and *Praon volucre* Haliday*.* Each species was housed in a separate insect-proof mesh cage (BugDorm: 60 x 60 x 60 cm) in controlled environment rooms (Fitotron) set to 20 ± 1 °C, 60 ± 5 % RH and a 16:8 photoperiod (light:dark). Two sweet pepper plants infested with *M. persicae* from the aphid culture were added to each parasitoid culture every week. Parasitoid cultures were maintained in a separate controlled environment room from the *M. persicae* culture, under the same conditions, to minimise cross-contamination. Plants were regularly watered by filling the bottom of the cage with 5mm of tap water whenever the soil surface was dry. Parasitoid cultures were inspected at least three times per week to assess whether the plants needed to be watered or additional aphid-infested pepper plants should be added.

2.3.3 Hyperparasitoids

A culture of *Asaphes suspensus* Walker were housed in separate insect proof mesh cages (BugDorm 47.5 x 47.5 x 47.5 cm). Hyperparasitoid cultures were maintained in controlled environment cabinets at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark) (Fitotron) separate to the primary parasitoid cultures to minimise cross-contamination. At least three sweet pepper plants containing aphid mummies were transferred from the primary parasitoid cultures to each of the hyperparasitoid cultures weekly. Plants were regularly watered by filling the bottom of the cage with 5 mm of tap water whenever the soil surface was dry. Hyperparasitoid cultures were inspected at least three times per week to assess whether the plants needed to be watered or additional parasitoid-infested pepper plants should be added.

2.4 Age-Standardised Cohorts

2.4.1 Aphids

Prior to using aphids in experiments, age-standardised cohorts were produced in controlled environment rooms (Fitotron) at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark). To standardise the aphids, adult peach potato aphids (*Myzus persicae*) cultured on sweet pepper were added to an uninfested sweet pepper plant and left to larviposit for 24 hours. After which, adults were removed and the offspring left to complete their development. Once these individuals reached reproductive maturity, a single standardised adult aphid was transferred to each experimental sweet pepper plant using a 000 paintbrush. Sweet pepper plants were then enclosed in a nylon mesh bag (18.3 cm x 12.6 cm Amazon, London) to limit

aphid movement. Aphids were left for 24 hours to larviposit. After this time, the adult aphid and all but one nymph were removed. The resulting offspring were considered age-standardised and could be grown to the required life-cycle stage for use in experiments. The number of adult aphids added to an uninfested sweet pepper plant was dependent on the quantity needed for the study.

2.4.2 Primary Parasitoids

Prior to using primary parasitoids in experiments, age-standardised cohorts were produced in controlled environment rooms (Fitotron) at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark). To standardise the parasitoids, a sweet pepper leaf infested with peach-potato aphids was placed into a glass Petri dish with female parasitoids of the same species for four hours. Following this, parasitoids were removed and disposed of and the sweet pepper leaf with parasitoid-exposed peach-potato aphids was placed onto a clean sweet pepper plant and housed in an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm). Once parasitised aphids mummified, they were removed with a 000 paintbrush and placed into a 960 ml ventilated plastic container (Pint-sized BugDorm, MegaView Science Co. Ltd, Taichung, Taiwan) with honey as a food source for emerging adults (Rowse Pure and Natural Honey, Wallingford, UK). Adults were identified as female before use in an experiment, typically within 48 to 72 hours after emergence. The number of aphids and parasitoids put into the petri dish was dependent on how many parasitoids were needed in the experiment.

2.4.3 Hyperparasitoids

Prior to using *Asaphes suspensus* in experiments, standardised cohorts were produced in controlled environment rooms (Fitotron) at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod of 16:8. To standardise hyperparasitoids, cohorts of age-standardised primary parasitoids were established as described in section 2.4.2. Mummified aphids were removed with a 000 paintbrush once parasitised and placed into a glass Petri dish with female hyperparasitoids for 4 hours. Hyperparasitoids were then removed and the mummies transferred to a ventilated 960 ml plastic container (Pint-sized BugDorm) with honey as a food source for emerging adults (Rowse Pure and Natural Honey) using a 000 paintbrush. Adults were identified as female before use in an experiment, typically within 48 to 72 hours after emergence. The number of aphid mummies and hyperparasitoids put into the Petri dish was dependent on how many hyperparasitoids were needed for an experiment.

CHAPTER 3

AN OBSERVATIONAL STUDY ON APHID, PRIMARY PARASITOID AND HYPERPARASITOID DEVELOPMENT

3.1 Introduction

Sweet pepper (*Capsicum* annum) is an economically important crop and in 2018, the UK produced 20,640 tonnes worth £40 million (Food and Agriculture Organization of the United Nations, 2021). Production of this crop is, however, negatively impacted by presence of insect pests (Table 3-1). The most common pest of UK sweet pepper crops is *M. persicae* (Hemiptera: Aphididae) (Fray et al., 2015). Aphids are a large group of Hemipteran insects characterised by piercing mouthparts that penetrate plant material to reach sugar rich phloem sap (Forbes, 1977). A small number of aphid species are considered economically important pests that impact a range of crop types globally, including sweet pepper (Morales-Hojas, 2017). Feeding by aphids directly damages the crop and causes distorted plant growth (Petitt and Smilowitz, 1982; Will and Vilcinskas, 2015) while indirect damage arises due to virus transmission or promotion of sooty mould growth due to excretion of sugar-rich honeydew (Petitt and Smilowitz, 1982).

Table 3-1: Common pests of sweet pepper crops in the UK (Cervantes, 2005; Fray et al., 2015; Sinaie et al., 2019; S. Wang et al., 2017; Wimmer et al., 2008; Zamani et al., 2007).

Invertebrate group	Pest species
Aphids (Insecta: Hemiptera: Aphidoidea)	Peach potato aphid (*Myzus persicae*) Melon cotton aphid (*Aphis gossypii*) Potato aphid (*Macrosiphum euphorbiae*) Foxglove aphid (*Aulacorthum solani*)
Mites (Arachnida: Trombidiformes)	Two spotted spider-mite (*Tetranychus urticae*)
Thrips (Insecta: Thysanoptera)	Western flower thrips (*Frankliniella occidentalis*) Onion thrips (*Thrips tabaci*)
Moth caterpillars (Insecta: Lepidoptera: Noctuidae)	Cabbage looper (*Trichoplusia ni)*

Many cropping systems traditionally rely on synthetic insecticides to control aphids and other invertebrate pests (Caballero-Lopez et al., 2011). However, an increasing number of these products are being withdrawn from the market due to concern regarding their impacts on both human and environmental health (Chowanski et al., 2014). Insecticides are potentially toxic to humans and some have been identified as being carcinogenic, neurotoxic and teratogenic (Alanja et al., 2014). Human health concerns specifically relate to the residual activity of some synthetic compounds (Sparks and Nauen, 2015), which are thought to cause acute health problems (Golge *et al.*, 2018). There are additional concerns related to the impact

that insecticides are thought to have on the environment and beneficial insects, particularly pollinators as a result of run-off contaminating soils, plants and aquatic habitats (Hladik, *et al.,* 2018). Sweet pepper crops are typically washed post-harvest to remove insecticide, however, it was been shown that residues often remain (Ahmed et al., 2011). Alongside human health, there are concerns related to the impact that insecticides are thought to have on the environment and beneficial insects, particularly pollinators (Hladik, *et al.,* 2018). Natural enemy populations target pests, such as aphids, and help to reduce pest pressure so it is counterproductive to reduce their presence through use of insecticides (Cloyd and Bethke, 2011). Overuse of insecticides can result in target pest(s) developing resistance (Foster et al., 2011). Insecticide resistance is a key example of rapid micro-evolution (Silva et al., 2012). Populations of *Myzus persicae* and the cotton aphid (*Aphis gossypii*), for example, have developed resistance to pyrethroid insecticides, rendering them inefficient in their control (Davies et al., 2007).

An alternative to relying on synthetic chemical insecticides involves using augmentation biological control as part of an integrated pest management (IPM) programme. This approach to biological control involves releasing mass-produced natural enemies with the aim of reducing a pest population within a crop (Collier and van Steenwyk, 2004). Parasitoid wasps are insects that complete the immature stages of their life cycle on or in a suitable host. The adult parasitoid will lay its egg in or on the host, the eggs then hatch and larvae feed on the host before pupation (Vinson, 1984). Most commercially available parasitoids belong to the order Hymenoptera and many species use aphids as their hosts (Ulber et al., 2010). There are six species of hymenopteran parasitoid commercially available to control aphid pests, which may be sold singularly or as a species mix (Biobest, 2019). The species mix is designed to aid in the control of a wide range of aphid species simultaneously (Boivin *et al.,* 2012). Each parasitoid species can be used in the control of a range of aphid species (Table 1-1). *Aphidius colemani* can successfully control 40 species of aphid (Sampaio *et al.,* 2008) and is commonly used throughout Europe in ornamental and protected vegetable crops (Vásquez *et al.,* 2006; Boivin *et al.,* 2011), including for *Myzus persicae* control in UK sweet pepper (Sampaio *et al.,* 2008; Fray *et al.*, 2015).

The efficacy of parasitoid based biological control programmes is threatened by the presence of hyperparasitoids (Gómez-Marco et al., 2015), which lay their eggs in or on their primary parasitoid hosts. Typical hyperparasitoid management in UK sweet pepper crops involves limiting primary parasitoid use and switching to broad spectrum insecticides to kill both hyperparasitoids and aphids (Fray et al., 2015). However, this approach results in the primary parasitoids also being killed (Edwards et al., 2008). This means the time and money spent on biological control will have been wasted (Jacobson, 2011). Occupying the fourth trophic level means that hyperparasitism is largely under-researched due to the nature of their

life cycle and how rearing them in the lab involves particular timings with regards to rearing aphids, primary parasitoids and plants simultaneously, resulting in fundamental gaps in the understanding of their biology and ecology (Aartsma et al., 2019b). With little knowledge of hyperparasitoid ecology it is difficult to control aphid hyperparasitoid pests in such a way as to allow continued use of primary parasitoids. It is unknown, for example, whether there is an optimum time after an aphid is parasitised, when a primary parasitoid is most likely to be attacked by a hyperparasitoid. This knowledge would enable further studies into the semiochemicals associated with parasitised aphid to which the hyperparasitoid may be responding. There remains a need for research on hymenopteran hyperparasitoids to develop practical solutions for their management within cropping systems.

A study was carried out with the aim of determining aphid, primary parasitoid and hyperparasitoid development under standardised laboratory conditions. This study aims to investigate whether parasitoids show a preference for the age/developmental stage of an aphid when selecting a host to larviposit in choice and no-choice experiments using *Aphidius colemani* as the study parasitoid and *M. persicae* as available host on sweet pepper plants. Parasitoid preferences, specifically in *A. colemani,* have been observed to favour intermediate aphid stages (Martinou and Wright, 2007). By testing host age preference through both choice and no-choice experiments, *Aphidius colemani* host selection may be illustrated as well as their host selection behaviour when there is only one choice available to them. This may give information on the ability of aphids to be parasitoid hosts at different developmental stages regardless of any apparent preference from the parasitoid.

3.2 Materials and Methods

3.2.1 Plant Propagation

Plants were propagated as described in Section 2.2.

3.2.2 Insects

Insects were reared as described in Section 2.3.

3.2.3 Age-Standardised Cohorts

Insect cohorts were age-standardised as described in Section 2.4.

3.2.4 Aphid Development Study

Twenty age-standardised peach-potato aphids (*Myzus persicae*) were used to produce nine sweet pepper plants containing a single first instar nymph. These first instar nymphs were then individually observed daily to monitor their development to reproductive maturity. Development through the immature nymph life cycle stages was monitored by the presence

of exuviae (i.e., moulted exoskeletons Fig. 3-1), which were removed using a 000 paintbrush and the date recorded. Aphids were restricted to their respective plants by enclosing them within nylon mesh bags (8.3 x 12.6 cm, Amazon, London). This study was carried out in a controlled environment room (Fitotron, Weiss Technik, UK Limited, Loughborough, UK) at 20 ± 1 °C, 60 ± 5% RH and a 16:8 (light:dark) photoperiod.

Figure 3-1: Exuviae cast from aphid nymph when developing to the next instar (Author's own).

3.2.5 Primary Parasitoid Development Study

As described in Section 2.3, 20 age-standardised *Myzus persicae* were used to produce 20 sweet pepper plants containing a single first instar nymph. These first instar nymphs were then observed daily to monitor their development as described in Section 2.4. Once an individual reached third instar it was placed into a glass Petri dish (100 mm x 15 mm) with one female *Aphidius colemani* adult aged between 24 hours and 48 hours. Third instar aphid nymphs were used for this study as it has previously been shown that *A. colemani* prefers this developmental stage when selecting a host (Zamani et al., 2006). They were observed until the parasitoid stings the aphid and oviposition occurs. This was recognised by the distinct positioning of the parasitoid whereby it bends its abdomen underneath its thorax and extends past the parasitoid's head and contacts the aphid's abdomen as the ovipositor pierces the aphid's exoskeleton to lay an egg inside of its abdomen. Once this occurs, the parasitoid was removed to prevent further oviposition events and the parasitised aphid transferred back to its sweet pepper plant. The parasitised aphid was then observed daily to record the number of days after parasitism that aphid 'mummification' (Fig. 3-2) and parasitoid adult emergence occurred (Fig. 3-3). This study was also repeated with *Aphidius ervi*, *A. matricariae, A. abdominalis, Ephedrus cerasicola* and *Praon volucre* simultaneously.

Figure 3-2: Mummified peach-potato aphid (*Myzus persicae*), parasitised by *Aphidius colemani* (Author's own).

Figure 3-3: (A) A newly emerged adult *Aphidius colemani* and (B) the mummified peach-potato aphid (*Myzus persicae*) from which it emerged (Author's own) and the bronze, rigid exoskeleton of the mummified peach-potato aphid showing where the adult parasitoid has eclosed and left a semi-circle emergence hole (Author's own).

3.2.6 Hyperparasitoid Development Study

Twenty developing *A. colemani* within *Myzus persicae* mummies were parasitised by age-standardised *Asaphes suspensus* and observed daily to record their development. Primary parasitoid development was repeated as described in Section 2.3. When the parasitised aphid mummified, it was transferred to a glass Petri dish (100 mm x 15 mm) with a with an assumed to be mated, female *A. suspensus* between 24 and 72 hours old. The mummy and hyperparasitoid were observed until the hyperparasitoid was observed to sting the mummy, identified as when the hyperparasitoid backs the end of her abdomen into the aphid and pierces it with the ovipositor. Hyperparasitoids were then removed to prevent further oviposition events and the mummy placed into an Eppendorf tube (1.5 ml) and observed daily

for morphological changes and hyperparasitoid emergence. This study was repeated as previously described with *Aphidius ervi* parasitised aphids.

3.2.7 Choice Experiment of Primary Parasitoid Preference for Aphid Age

Age standardised reproducing adult *M. persicae* were places on individual sweet pepper plants which resulted in six young sweet pepper plants with a single first instar *M. persicae* on by placing one adult onto the plant until they produce offspring and then removing the adult and all but one nymph. The experiment required five *M. persicae* at different developmental stages (1st instar, 2nd instar, 3rd instar, 4th instar and an adult), therefore the dates at which the reproducing adults were put onto the plants was staggered by one day (24 hours). The first adult put onto a pepper plant was to produce a nymph that would be observed until reaching the mature, reproducing adult stage, at which point it would be used in the experiment. On the following day (24 hours later), the adult put onto a pepper plant was used to produce a nymph that would be observed until reaching the immature, non-reproductive adult stage. The adult put onto a pepper plant another 24 hours later would produce an offspring that would be observed until reaching 4th instar when it would then be used in the experiment, and so forth. Aphids were restricted to their respective plants by enclosing them within nylon mesh bags (18.3 x 12.6 cm). Once there were six individual aphids, each of a different developmental stage, they were removed from their pepper plants with a 000 paintbrush and placed into a single Petri dish with one unfed female *A. colemani* aged between 24 hours and 48 hours.

The parasitoid was left in the Petri dish with the five aphids each of a different developmental stage for a further 1 hour. The parasitoid was then removed from the Petri dish and the aphids returned to their individual plants using a 000 paintbrush and were observed daily to record whether they mummified and on what date so that a time from parasitism to mummification could be calculated. The mummy was placed into an Eppendorf tube and observed daily until the parasitoid emerged so that the date of emergence and total development time could be calculated. This study was carried out in a controlled environment room (Fitotron) at 20 ± 1^{o}C, 60 ± 5% RH and a 16:8 (light:dark) photoperiod 20 times.

3.2.8 No-Choice Experiment of Primary Parasitoid Preference for Aphid Age

Age standardised reproducing adult *M. persicae* were used to produce six young sweet pepper plants with a single first instar *M. persicae* on by placing one adult onto the plant until they produce offspring and then removing the adult and all but one nymph. The experiment required 20 *M. persicae* at different developmental stages (1st instar, 2nd instar, 3rd instar, 4th instar, non-reproducing adult and reproducing adult), therefore the dates at which the reproducing adults were put onto the plants was staggered by one day (24 hours). The first

adult put onto a pepper plant was to produce a nymph that would be observed until reaching the mature, reproducing adult stage, at which point it would be used in the experiment. On the following day (24 hours later), the adult put onto a pepper plant was to produce a nymph that would be observed until reaching the immature, non-reproductive adult stage. The adult put onto a pepper plant another 24 hours later would produce an offspring that would be observed until reaching 4th instar when it would then be used in the experiment, and so forth. Aphids were restricted to their respective plants by enclosing them within nylon mesh bags (18.3 x 12.6 cm). Once there were six individual aphids, each of a different developmental stage, they were each removed from their pepper plants with a 000 paintbrush and placed into separate Petri dishes with one unfed female primary parasitoid *Aphidius colemani* aged between 24 hours and 48 hours. The Petri dish would, therefore, have five *M. persicae* at the same developmental stage with one female *A. colemani.*

The parasitoid was left in the Petri dish with the aphids for a further 1 hour. The parasitoid was then removed from the Petri dish and the aphids returned to their individual plants using a 000 paintbrush and were continued to be observed daily to record whether they mummified and on what date so that a time from parasitism to mummification could be calculated. The mummy was placed into an 2ml Eppendorf tube and observed daily until the parasitoid emerged so that the date of emergence and total development time could be calculated (Fig. A). This study was carried out in a controlled environment room (Fitotron) at 20 ± 1^{o}C, 60 ± 5% RH and a 16:8 (light:dark) photoperiod. This experiment was replicated 20 times.

3.2.9 Statistical Analysis

Underlying data distributions for each development study (except aphid development) were first tested using Shapiro-Wilk tests to establish whether they violate parametric assumptions. As data were found to be non-Gaussian, Mann-Whitney *U*-tests were used to evaluate the difference between the median development time of *Aphidius colemani* and *Aphidius ervi;* comparing the overall development time, from oviposition to emergence, as well as the time from oviposition to aphid mummification and from mummification to adult emergence. The time differences between each developmental stages across the six species were calculated using the Dunn Test. This test was also used to evaluate the median difference in development time of *Asaphes suspensus* when developing on the two parasitoid hosts, *A. colemani* and *A. ervi.* All statistical analyses were performed with R version 4.0.4 (R Core Team, 2021).

3.3 Results

3.3.1 Aphid Development

The mean number of days that 20 *M. persicae* spent at each development stage was calculated (Fig. 3-4). The mean number of days spent as a first instar nymph was 1.78 ± 0.21, second instar nymph was 1.33 ± 0.17, third instar nymph was 1.44 ± 0.18, fourth instar nymph was 1.78 ± 0.15 and the mean number of days spent in the fifth developmental stage (non-reproducing adult) was 1.11 ± 0.20. The overall development time was a mean of 7.44 ± 0.18 days before becoming a reproducing adult.

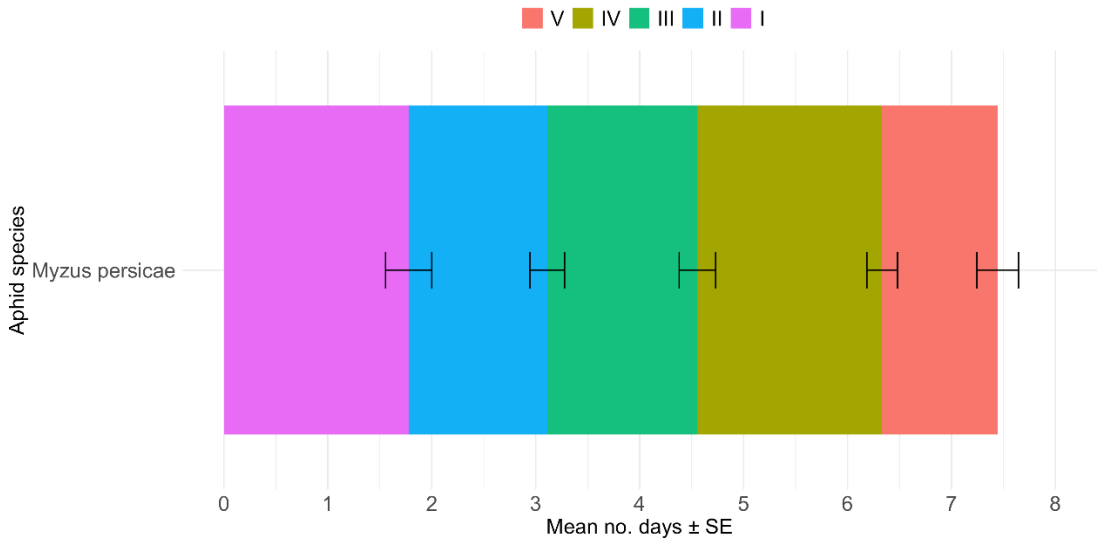


Figure 3-4: Peach-potato aphid (*Myzus persicae*) development time from birth to reproducing adult.

3.3.2 Parasitoid Development

A. colemani, A. ervi, A. matricariae, A. abdominalis, E. cerasicola and *P. volucre* each successfully parasitised 20 third instar *M. persicae* during this study (120 parasitised aphids in total). There was a significant difference in the total development time from oviposition to adult emergence between the six species (X^2 = 80.28, df = 5, $P < 0.01$, n = 120). *Praon volucre* had the longest development time with a mean of 17.4 days. *Aphidius abdominalis* was the second longest mean development time of 15.45 days, next was *E. cerasicola* with a mean development time of 14.7 days, then *A. matricariae* with a mean development time of 13.5 days with *A. colemani* and *A. ervi* with the same and shortest mean development time of 13.2 days. There was also a significant difference observed between the time each species spent developing into a mummy from oviposition (X^2 = 45.4 , df = 5 , $P < 0.01$, n = 120) with *P. volucre* taking an mean time of 9.25 days, *A. abdominalis* with a mean of 8.6 days, *E. cerasicola* with a mean of 8.55 days, *A. matricariae and A. colemani* both with a mean of 7.8

days and finally *A. ervi* with the shortest mean development time from oviposition to mummification of 7.5 days. There was also a significant difference observed between the time each species spent developing from mummification to adult emergence ($X^2 = 70.64$, df = 5 , $P < 0.01$, $n = 120$) with *P. volucre* showing the longest development time of 8.15 days followed by *A. abdominalis* taking a mean time of 6.85 days, *E. cerasicola* with a mean of 6.15 days, *A. ervi* and *A. matricariae* both taking a mean of 5.7 days and the shortest mean development time from mummification to adult emergence being *A.* colemani at 5.4 days (Fig. 3-5). There was no significant difference between total development time for *E. cerasicola, A. abdominalis* and *A. matricariae*. Development time at all stages is not significantly different between the three *Aphidius* species, *A. colemani, A. ervi* and *A. matricariae*.

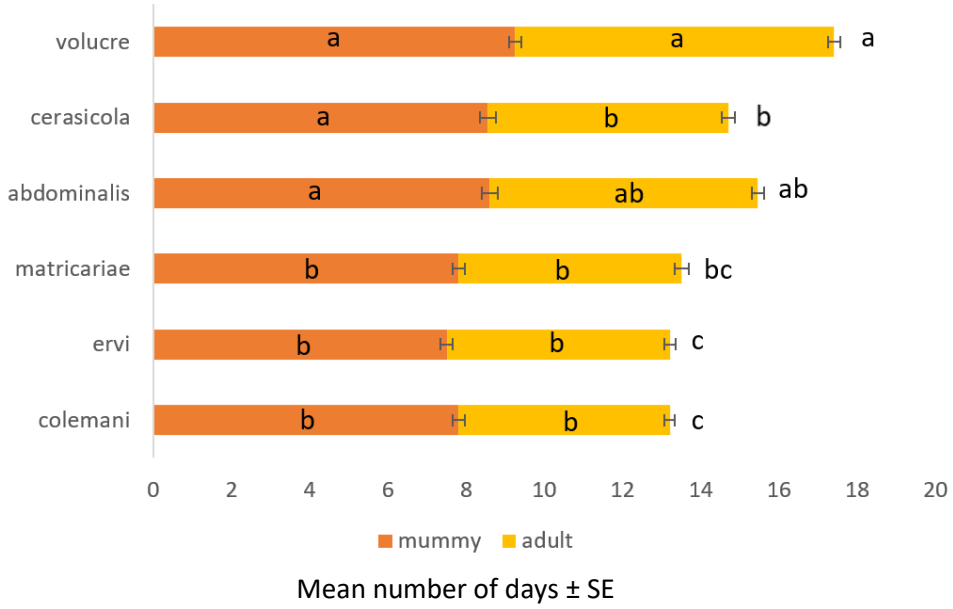


Figure 3-5: Development time of the six primary parasitoid species (*n* = 120). Error bars represent standard error of the mean (SE). Bars capped with letters to show differences in mean between the total development time and oviposition to adult emergence across all six species. Letters in the centre of the orange portion of the bars show differences between time from oviposition to mummification across all six species. Letters in the centre of the yellow portion of the bars show differences between time from mummification until adult emergence across all six species and the letters topping the overall bars show differences in total time from oviposition until adult emergence. These were calculated using the Dunn Test.

3.3.3 Primary Parasitoid Preference for Aphid Age

In the choice experiment, comparing the chosen developmental stage of *A. colemani* when presented with five *M. persicae* each at different developmental stage, third instar was the most frequently chosen developmental stage with 11 out of the available 20 aphids being

parasitoids (Fig. 3-6). Six fourth instar aphids were successfully parasitised, four second instar and two adult aphids were parasitised. No first instar aphids were successfully parasitised in this choice experiment. Twenty three percent of the aphids used in this experiment were parasitised (five per Petri dish). Three Petri dishes had two aphids parasitised together. An individual *Aphidius colemani* successfully parasitised a third and fourth instar within the same Petri dish twice. A fourth and second instar aphid was parasitised within the same Petri dish by a single *Aphidius colemani* also.

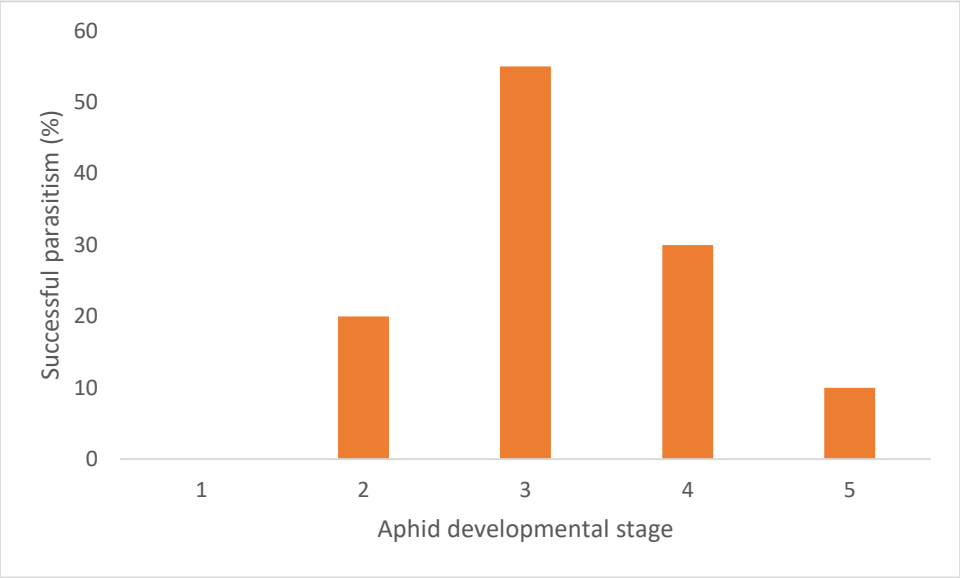


Figure 3-6: Percentage successful parasitism of *Myzus persicae* at each developmental stage (1 = first instar, 2 = second instar, 3 = third instar, 4 = fourth instar and 5 = adult) by *Aphidius colemani* when given a choice of all five developmental stages.

In the no-choice experiment, comparing the number of successful parasitism of *A. colemani* on *M. persicae* at different developmental stages when presented with no choice, the highest successful rate of parasitism was seen in third instar aphids where 66.67 % of the aphids were successfully parasitised, followed by 60 % of the fourth instar aphids and the least successful rate of parasitism seen in first instar aphids where only 10% were successfully parasitised out of the 20 replicates (Fig.3-7).

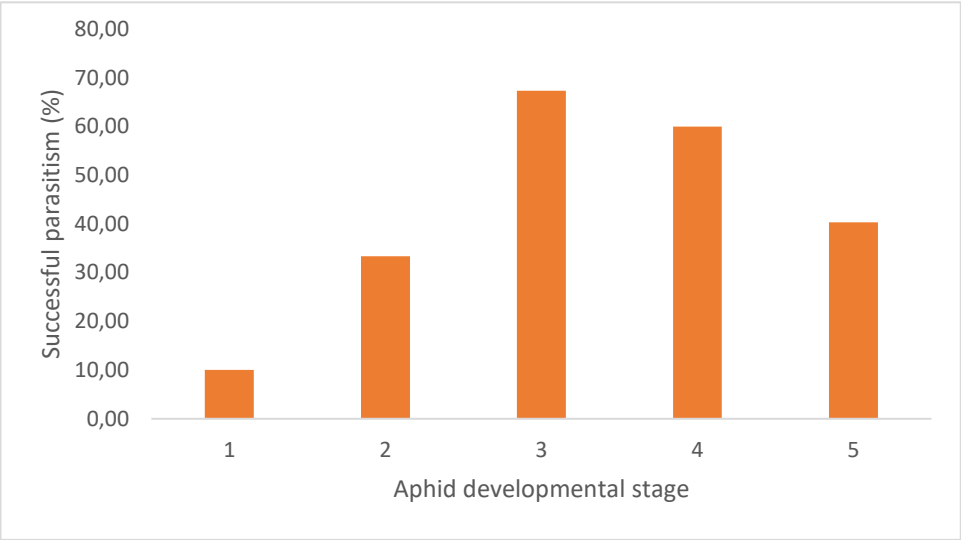


Figure 3-7: Percentage successful parasitism of *Myzus persicae* at each developmental stage (1 = first instar, 2 = second instar, 3 = third instar, 4 = fourth instar and 5 = adult) by *Aphidius colemani* when only given a choice of one stage.

3.3.4 Hyperparasitoid Development

Asaphes suspensus successfully parasitised 120 third instar *Myzus persicae* in this study. The aphids were evenly split into six groups of twenty, each group was previously parasitised by a different primary parasitoid; *Aphidius colemani, A. ervi, A. matricariae, A. abdominalis, E. cerasicola* or *P. volucre*. There was no significant difference observed in the development time of the hyperparasitoid on the different hosts ($X^2 = 0.77$, df = 5, *P>0.5, n* = 120) (Fig. 3-8). *Asaphes suspensus* development time ranged from a mean of 17.9 days with *A. matricariae* as host to 18.15 with *A. evri* and *E. cerasicola* as hosts.

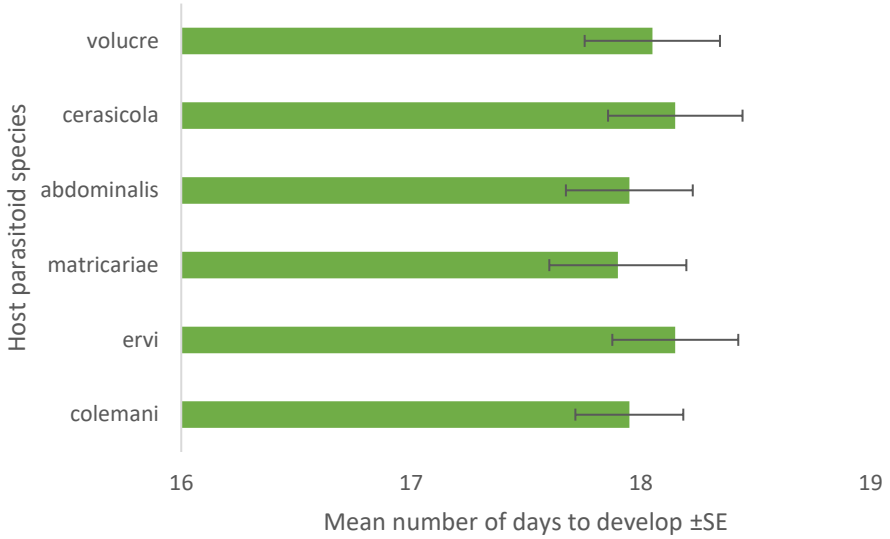


Figure 3-8: Development time of *Asaphes suspensus* when parasitising six different parasitoid hosts, *Aphidius colemani, A. ervi, A. matricariae, Aphidius abdominalis, Ephedrus cerasicola* and *Praon volucre* showing no significant difference in the mean number of days from oviposition to adult emergence.

3.4 Discussion

3.4.1 Aphid Development

The development of the peach-potato aphid (*Myzus persicae*) took a mean of 7.44 ± 0.18 days from first instar to reproductive adult, with just over 1 day being spent at each instar. This data corroborates similar studies reporting development times for this species of approximately one week under similar conditions to those used in this study (Blackman, 2009).

3.4.2 Parasitoid Development

Aphidius colemani, Aphidius evri, Aphidius matricariae, Aphidius abdominalis, Ephedrus cerasicola and *Praon volucre* are the most commonly used primary parasitoids in aphid control in sweet pepper cropping systems in the UK (Fray et al., 2015). These species can be bought together in a species mix for growers to release for the control of a wide range of pests (Boivin *et al.*, 2011). Here, the development time of these species was studied under standardised laboratory conditions. All six parasitoid species were able to parasitise third instar peach-potato aphid, which was expected as they are known parasitoids of this aphid species (Zamani et al., 2006) and are known to have a higher parasitism success rate in this aphid developmental stage than in the other stages (Perdikis *et al.*, 2004; Kumar *et al.*, 2019). The total development time ranged from just over 13 days in all three *Aphidius* species to 17.4 days in *P. volucre.* The development observations corroborate previous research on these species where a development time of approximately 13 days has been observed in *Aphidius*

species (Perdikis *et al.*, 2004; Henry *et al.*, 2005) and *P. volucre* is known to take longer to develop (Sigsgaard, 2000).

Typical biological control practice in UK protected sweet pepper crops is to release these parasitoid species early in the year on a weekly basis as an initial preventative approach to aphid control; releasing at least 0.15 individuals per m^2. Many growers will set up sticky traps or pan traps to monitor aphid populations and once they've been detected the number of parasitoids released is increased to 0.5-1 m^2/week for at least another three weeks (Goh *et al.*, 2001). This application method is suitable for the range of parasitoid species. Though this study showed a significant difference in total development time between the six primary parasitoid species, a difference of just over four days is unlikely to require any species-specific techniques to be considered as a direct result of this observation, especially as all six species demonstrated successful parasitism of *M. persicae.*

3.4.3 Primary Parasitoid Aphid Age Preference

Aphidius colemani showed a preference for parasitising *Myzus persicae* when at third instar over other developmental stages. When not given a choice and being presented with an individual *M. persicae, A. colemani* parasitised 67.34 % of the third instars presented to it which was the highest rate of successful parasitism across the developmental stages (60% of fourth instars, 40.33 % of adults, 33.33 % of second instars and 10 % of first instars). It was to be expected, then, that when given a choice of five different developmental stages, *A. colemani* parasitised 55 % of the third instar aphids, the highest proportion of successful parasitism across the different developmental stages offered (30 % of fourth instars, 20 % of second instars, 10 % of adults and no first instars). The preference for third instar has also been observed as a preferable size and life stage for parasitoid oviposition in a study by Sampaio *et al.* testing different sized *Aphis gossypii* Glover, *Brevicoryne brassicae* L., *M, persicae, Rhopalosiphum maidis* Fitch and *Schizaphis graminum* Rondani as available hosts to *Aphidius colemani* (Sampaio et al., 2008). Colinet *et al.* (2005) also observed *A. ervi* displaying a preference for the intermediate developmental stages of *M. persicae,* suggesting the results of this study showing *A. colemani* to favour second to fourth instar, with a preference for third, may also apply to other parasitoid species (Colinet *et al*., 2005). Larger hosts, such as adult aphids, may seem the most suitable for their larger size and, therefore, more resource for the hatching parasitoid to feed on. However, *Aphidius colemani* is a koinobiont parasitoid, meaning its host continues to feed and grow after oviposition until mummification occurs approximately a week later, that with the fact that adult aphids are better able to defend themselves may explain these results (Stary, 1975). Looking at the results of the aphid development study, *M. persicae* reaches the adult stage around 3 days from being

third instar, so the aphid is likely to be fully grown with ample resources for the parasitoid once it hatches.

Colinet *et al.* (2005) also studied the fitness of the emerging adult parasitoids in relation to the developmental stage of the host. They observed no differences in parasitoid fitness so it can be presumed that, though the parasitoids may prefer hosts of this developmental stage and size, there isn't a negative impact on fitness when they parasitised aphids of other developmental stages. It has been observed, in a study by Chau and Mackauer, that hymenopteran parasitoid *Monoctonus paulensis* Ashmead (Braconidae, Aphidiinae) has a preference for second instar *Acyrthosiphon pisum* Harris (Hemiptera, Aphididae) over third instar *Sitobion avenae* Fabricius (Braconidae, Aphidiinae) when given a choice of both (Chau and Mackauer, 2001). In which case, host species may have a stronger influence on parasitoid host selection than developmental stage.

In conclusion, *Aphidius colemani* shows a preference for third instar aphids when given a choice between first, second, third and fourth instar and a reproducing adult and successfully parasitises the intermediate aphid developmental stages much more frequently than adults or first instar aphids when given a choice. However, the no-choice experiments showed that *A. colemani* can successfully parasitise the less-preferred aged aphids which may mean that parasitoids are able to successfully parasitise early-stage aphids as populations are trying to establish within cropping systems. Parasitoids such as *A. colemani* may, therefore, be able to establish a population within cropping systems even when aphids are not yet well established within the crop.

3.4.4 Hyperparasitoid Development

The efficacy of parasitoid wasps as biological control agents is threatened by the presence of hyperparasitoids (Gómez-Marco et al., 2015). In a 2015 review, 16 representatives involved in sweet pepper production were interviewed and all were familiar enough with hyperparasitism to identify its presence and recognised this as a threat to sweet pepper crops where there is reliance on parasitoids for aphid control (Fray et al., 2015). Typical practice, once hyperparasitism is detected within a sweet pepper crop, is to cease releases of aphid parasitoid and instead to switch to use an insecticide to kill the hyperparasitoids and aphids. Growers are aware that this results in a decrease in the parasitoid population that they paid for and worked to release into the system.

Asaphes suspensus is one of the most common hyperparasitoid species found in protected sweet pepper crops in the UK (de Boer *et al*., 2019). *Asaphes suspensus* was able to hyperparasitise all six primary parasitoid species which was expected as *A. suspensus* is a known parasitoid of these species (de Boer et al., 2019). There was no difference shown between the development time of *A. suspensus* when parasitising the six hosts, taking 17.9

days to fully develop in *A. matricariae* to 18.15 days in *E. cerasicola* and *A. ervi*. The combination of the parasitoid and hyperparasitoid development studies illustrate no difference in the development of *A. suspensus* when parasitising the six host species. If the hyperparasitoid species commonly found in sweet pepper glasshouses, *A. suspensus,* has a preference for which of the six commercially available Hymenopteran parasitoids that they will parasitise, then there is potential for growers to alternate between the parasitoid species that they release into their crop (Cusumano et al., 2020). This may result in the hyperparasitoid having a reduced effect on the primary parasitoid population. However, this study shows that changing between the six parasitoid species, or using a combination of these species, within cropping systems will not influence the ability for *A. suspensus* to reproduce.

3.5 Further Work

This study has provided integral information on the development of the project's study species which informs the methodology of further experimentation as well as giving an insight to the fundamental ecology of the species. By studying the parasitoid host age preferences, a deep level of understanding into the details of host selection has been given. As this project aims to develop research into aphid hyperparasitoids, continuing this research into studying the impact that hyperparasitoids have on the ability for a primary parasitoid ability to establish populations and to select aphid hosts.

CHAPTER 4

DOES HYPERPARASITOID EXPOSURE INFLUENCE OVIPOSITIONSUCCESS IN SIX COMMERCIALLY AVAILABLE APHID PRIMARY PARASITOIDS?

4.1 Introduction

Conventional cropping systems heavily rely on synthetic insecticides to control aphids and other invertebrate pests (Caballero-Lopez *et al*., 2011). An alternative to using synthetic chemical insecticides for pest control is augmentation biological control as part of a wider integrated pest management (IPM) programme. Sweet pepper production in the UK uses IPM strategies that emphasise use of biological control agents over synthetic insecticide applications. For example, in 2019 538 hectares of sweet pepper crops in the UK received insecticide applications against 6,718 hectares that were treated with biological control agents (Ridley et al., 2019). Six commercially available hymenopteran primary parasitoids are widely used for aphid control in protected horticulture IPM programmes (Sampaio *et al.,* 2008; Fray *et al.*, 2015). However, their success in UK sweet pepper crops is severely impacted by hyperparasitoids from the *Asaphes* genus (Gómez-Marco et al., 2015; de Boer *et al*., 2019). Typical hyperparasitoid management involves switching from primary parasitoids to using aphid predators such as *Aphidoletes* or, more commonly, broad spectrum insecticides that kill both hyperparasitoids and aphids (Fray *et al.*, 2015). This approach results in beneficial organisms being killed and emergence of secondary pests (Edwards *et al.*, 2008).

Hyperparasitism is an under-researched phenomenon, resulting in fundamental gaps in the understanding of hyperparasitoid biology and ecology (Aartsma et al., 2019b). With little knowledge of their ecology, it is difficult to manage aphid hyperparasitoids in such a way as to allow economically viable use of primary parasitoids in IPM programmes. This study aimed to contribute to wider knowledge of hyperparasitoid ecology by determining whether primary parasitoid oviposition success is influenced by exposure to adult hyperparasitoids or hyperparasitised mummies. It also aimed to elucidate whether primary parasitoid oviposition behaviour was influenced by hyperparasitoid-derived chemical cues. It is hypothesised that the presence of *Asaphes suspensus* adults and mummies significantly reduce the oviposition success of six commercial primary parasitoid species in the aphid *Myzus persicae.* It is also hypothesised that washing the leaves after adult hyperparapsitoid exposure or whilst hyperparasitised mummies are on the leaf would result in no reduction in primary parasitoid oviposition success. It was hypothesised that hyperparasitoid chemical cues could be reapplied to clean leaves after washing them off leaves exposed to adult hyperparasitoids or with hyperparasitised mummies on to reduce primary parasitoid oviposition success.

Greater understanding of primary parasitoid-hyperparasitoid interactions will aid in developing practical solutions for hyperparasitoid management in protected cropping systems.

4.2 Materials and Methods

4.2.1 Plants

Plants were propagated as described in Section 2.2.

4.2.2 Aphids

Aphid culturing and creation of standardised cohorts was as described in Sections 2.3.1 and 2.4.1**.**

4.2.3 Primary Parasitoids

Primary parasitoids were reared and standardised as described in Sections 2.3.2 and 2.4.2.

4.2.4 Hyperparasitoids

Hyperparasitoids were reared and standardised as described in Sections 2.3.3 and 2.4.3.

4.2.5 Effect of Adult Hyperparasitoid Trails on Primary Parasitoid Oviposition Success

To determine whether adult hyperparasitoid presence impacts primary parasitoid oviposition success, primary parasitoids were presented with age-standardised aphid colonies on sweet pepper leaves previously exposed to *A. suspensus*. This was achieved by restricting age-standardised and potentially mated adult female *A. suspensus* onto a single pepper plant leaf at BBCH (Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie) growth stage one with a clip cage (36.5 x 25.4 x 9.5 mm, BioQuipp Products, Inc. Compton, CA 90220, United States) for one hour inside an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) in a controlled environment room (Fitotron) at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark). The number of hyperpasitoids used was either zero (control), one, two, four or eight individuals. To put the hyperparasitoids into the clip cages, they would first be removed from the standardised cohort using a pooter (Pocket Pooter, Watkins & Doncaster, UK) and placed into a freezer at -20 ˚C for up to three minutes to reduce their movement. They were then removed and ten age standardised third instar *M. persicae* were transferred onto the same leaf using a 000 paintbrush. The plant was then transferred to an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) for ten minutes before checking that they had settled on the leaf and then five age-standardised and potentially mated adult female primary parasitoids were released into the cage for one hour.

Each aphid infested experimental plant was exposed to one primary parasitoid species and replications for each primary parasitoid species was carried out simultaneously. Primary parasitoids were removed from the cage with a pooter (Watkins & Doncaster) and the ten aphids on the sweet pepper plant were transferred to a fresh sweet pepper plant using a 000 paintbrush. This plant was secured within an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) and observed daily from the sixth day post-primary parasitoid exposure until adult primary parasitoid emergence (the earliest aphids have been shown to mummify in prior experiments following oviposition by the study species of primary parasitoids) to record how many aphids developed into mummies and therefore considered successfully parasitised. Primary parasitoids were left to develop and emerge from the aphid mummy to be weighed (Mettler Toledo Balance XPR10/M, Columbus, Ohio, United States), sexed (ovipositor presence/absence and antennal length) and measured (head width at the widest point, body length from the top of the head to the tip of the abdomen and left hind leg length) under a stereoscopic microscope (Microtec HM-3, Tec Microscopes Ltd, Somerset, UK) in order to monitor host effects on adult parasitoids. This experiment was carried out within a controlled environment room (Fitotron) maintained at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark) and replicated twenty times for each primary parasitoid species.

4.2.6 Effect of Hyperparasitised Mummy Presence on Primary Parasitoid Oviposition Success

To determine whether the presence of hyperparasitoid larvae within aphid mummies (referred to from this point as hyperparasitised mummies) on a leaf impacts primary parasitoid oviposition behaviour, commercially available primary parasitoids were presented with ten age-standardised aphids on a sweet pepper leaf containing *A. suspensus* mummies. This was achieved by securing age-standardised hyperparasitised mummies to a single pepper plant leaf at BBCH growth stage one a week after the hyperparasitoid egg was laid inside the aphid mummy. Attachment was by pinning the mummy to the leaf through the first thoracic segment to prevent damaging the developing hyperparasitoid within using entomological pins (Continental S/Steel Nylon Head Pins No.3, Watkins & Doncaster, Leominster HR6 0RG). Ten third instar *M. persicae* were transferred onto the same leaf using a 000 paintbrush within the next five minutes. The number of hyperparasitoid mummies used was either zero (control), one, two, four or eight. The plant was then transferred to an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) and five age-standardised and potentially mated adult female primary parasitoids were released into the cage within the next five minutes to be left for one hour. Each aphid infested experimental plants was only exposed to one species of primary parasitoid and replications for each primary parasitoid species were carried out simultaneously. Primary parasitoids were then removed from the cage with a pooter (Watkins & Doncaster) and the ten aphids on the sweet pepper plant were transferred to a fresh sweet

pepper plant using a 000 paintbrush. This plant was secured in an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) and monitored from the sixth day post-primary parasitoid exposure until adult primary parasitoid emergence to record how many of the ten aphids were successfully parasitised, as identified by development of an aphid mummy. Primary parasitoids were left to develop and emerge from the aphid mummy to be weighed (Mettler Toledo Balance XPR10/M), sexed (ovipositor presence/absence and antennal length) and measured using an eyepiece graticule (head width at the widest point, body length from the top of the head to the tip of the abdomen and left hind leg length) under a stereoscopic microscope (Microtec HM-3) in order to monitor host effects on adult parasitoids. This experiment was carried out within a controlled environment room (Fitotron) maintained at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark) and replicated twenty times for each primary parasitoid species.

4.2.7 Removal of Oviposition-Deterrent Cues Associated with Adult Hyperparasitoid Presence

To determine whether washing sweet pepper leaves after exposure to adult hyperparasitoids impacts primary parasitoid oviposition success, primary parasitoids were presented with ten age-standardised aphids on a sweet pepper leaf previously exposed to *Asaphes suspensus* adults. This was achieved by repeating the steps outlined in Section 2.2 carried out with an additional step of washing the leaf once the adult hyperparasitoids were removed from the clip cage. Washing was carried out by submerging the leaf in distilled water (Du et al., 1996), whilst still attached to the plant, within a 1.5 ml Eppendorf tube (Sarstedt, Numbrect, Germany) and rotating it for 30 seconds. The plant was then placed into an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) and left to air dry for 30 minutes before transferring ten third instar *M. persicae* onto the leaf and following the final steps outlined in Section 4.2 by placing the aphid infested plant in a mesh cage with five adult females of one of the primary parasitoid species for one hour. This experiment was carried out within a controlled environment room (Fitotron) maintained at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark) and replicated twenty times for each primary parasitoid species.

4.2.8 Removal of Oviposition-Deterrent Cues Associated with Hyperparasitoid Mummy Presence

To determine whether washing sweet pepper leaves with hyperparasitised mummies impacts primary parasitoid oviposition success, primary parasitoids were presented with age-standardised aphid colonies on sweet pepper leaves containing *A. suspensus* mummies. This was achieved by repeating the steps outlined in Section 2.3 with an additional step of washing the leaf and attached hyperparasitoid mummies. Washing was carried out by submerging the leaf and hyperparasitoid mummies in distilled water (Du et al., 1996), whilst still attached to

the plant, within a 500 ml plastic tub (RelianceUK, Yorkshire, UK) and rotating it for 30 seconds. The plant was then placed into an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) and left to air dry for 30 minutes before transferring ten third instar *M. persicae* onto the leaf and following the final steps outlined in Section 4.3.1 by placing the aphid infested plant in a mesh cage with five adult females of one of the primary parasitoid species for one hour. This experiment was carried out within a controlled environment room (Fitotron) maintained at 20 ± 1 ˚C, 60 ± 5 % RH with a 16:8 photoperiod (light:dark) and replicated twenty times for each primary parasitoid species.

4.2.9 Removal and Re-Application of Oviposition-Deterrent Cues Associated with Adult Hyperparasitoid Exposure

To determine whether washing sweet pepper leaves with distilled water after adult hyperparasitoid exposure and then reapplying the solution to clean pepper plant influences primary parasitoid oviposition success, *A. colemani, A. abdominalis, E. cerasicola* and *P. volucre* were presented with age-standardised aphid colonies on sweet pepper leaves previously exposed to *A. suspensus* adults. Both *A.ervi* and *A. matricariae* were excluded from this experiment as they exhibited no difference in the oviposition success of the three *Aphidius* species tested and so one species, *A. colemani,* from this genus was used here as it is the most commonly used species for aphid control (Sampaio *et al.,* 2008; Fray *et al.*, 2015). To do this, zero, two or eight age-standardised and potentially mated adult female *A. suspensus* onto a single leaf of a pepper plant at BBCH growth stage one with a clip cage (36.5 x 25.4 x 9.5 mm, BioQuip Products, Inc.) for one hour inside an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm). The clip cage and hyperparasitoids were then removed and the leaf submerged in distilled water within 1.5 ml Eppendorf tube and rotated for 30 seconds (Du et al., 1996). The distilled water used to clean the leaf after adult hyperparasitoids had been present remained within the Eppendorf tube and was then painted on to a single leaf of a fresh sweet pepper plant at BBCH growth stage one using a clean 000 paintbrush; applying the water to the leaf from stem to tip of the leaf, topside and underside. Re-application of the water-leaf extraction occurred within five minutes of washing the leaf. Ten age-standardised third instar *M. persicae* were placed onto the same leaf using a clean 000 paintbrush over the next five minutes and the plant transfer to an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) with five age-standardised and potentially mated adult female primary parasitoids into the cage for one hour.

Once the primary parasitoids were removed from the cage with a pooter (Watkins & Doncaster) the aphids were transferred to a fresh sweet pepper plant using a 000 paintbrush and secured within another insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) and observed on the sixth day then daily from that point until adult primary parasitoid emergence,

to record how many of the ten aphids were successfully parasitised, as identified by the development of aphid mummification. Once the adult primary parasitoids emerge from the aphid mummy they were weighed (Mettler Toledo Balance XPR10/M), sexed (ovipositor presence/absence and antennal length) and measured (head width at the widest point, body length from the top of the head to the tip of the abdomen and left hind leg length) under a stereoscopic microscope (Microtec HM-3) in order to monitor host effects on adult parasitoids and replicated twenty times for each primary parasitoid species.

4.2.10 Removal and Re-Application of Anti-Oviposition Cues Associated with Hyperparasitoid Mummy Exposure

To determine whether washing sweet pepper leaves with distilled water after hyperparasitoid mummy exposure and then reapplying the solution to clean pepper plant impacts primary parasitoid oviposition success, *A. colemani, A. abdominalis, E. cerasicola* and *P. volucre* were presented with age-standardised aphid colonies on sweet pepper leaves previously with *A. suspensus* mummies within an aphid mummy on them. This was achieved by securing zero, two or eight age-standardised hyperparasitoid mummies to a single pepper plant leaf at BBCH growth stage one a week after hyperparasitoid oviposition. Attachment was by pinning the mummy to the leaf through the first thoracic segment to prevent damaging the developing hyperparasitoid within using entomological pins (Continental S/Steel Nylon Head Pins No.3, Watkins & Doncaster). The leaf with the attached mummies was then submerged in distilled water within a 500ml plastic tub (RelianceUK) and rotated for 30 seconds (Du et al., 1996). The distilled water used to clean the leaf and mummies remained within in the tub and was then painted on to a single leaf of a fresh sweet pepper plant at BBCH growth stage one using a clean 000 paintbrush; applying the water to the leaf from stem to tip of the leaf, topside and underside. Ten age-standardised third instar *M. persicae* were placed onto the same leaf of the sweet pepper plant using a clean 000 paintbrush within the next five minutes. The pepper plant was transferred to an insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm) with five age-standardised and potentially mated adult female primary parasitoids and left for one hour. Once the primary parasitoids were removed from the cage with a pooter (Watkins & Doncaster), the aphids were transferred to a fresh sweet pepper plant using a 000 paintbrush and secured within another insect proof mesh cage (BugDorm: 47.5 x 47.5 x 47.5 cm). The aphids were observed from the sixth day and then daily from this point until adult parasitoid emergence, to record how many of the ten aphids were successfully parasitised, as identified by the development of aphid mummification. Once the adult primary parasitoids emerged from the aphid mummy they were weighed (Mettler Toledo Balance XPR10/M), sexed (ovipositor presence/absence and antennal length) and measured (head width at the widest point, body length from the top of the head to the tip of the abdomen and left hind leg

length) under a stereoscopic microscope (Microtec HM-3) in order to monitor host effects on adult parasitoids.

4.2.11 Statistical Analysis

Primary parasitoid oviposition success in each experiment was analysed using generalised linear models (GLMs) fitted with quasi-Poisson distributions to account for overdispersion. Both primary parasitoid and number of hyperparasitoids were used as fixed factors. Post-hoc mean separation was determined for number of hyperparasitoids using Tukey's Honestly Significant Difference test. Primary parasitoid morphological measurements in each experiment were analysed using GLMs fitted with Gaussian distributions. Both primary parasitoid and number of hyperparasitoids were used as fixed factors. Post-hoc mean separation was determined for number of hyperparasitoids using Tukey's Honestly Significant Difference test. All statistical analyses were carried out in R (version 4.1.3) (R Core Development Team, 2022).

4.3 RESULTS

4.3.1 Effect of Adult Hyperparasitoid Trails on Primary Parasitoid Oviposition Success

Hyperparasitoid presence had a negative impact on primary parasitoid oviposition success across all six species tested in no choice dose response experiments (generalised linear model with quasi-Poisson distribution: $F = 1.3446$; $d.f. = 4$, $P < 0.001$) (Fig. 4-1). All primary parasitoid species, except *E. cerasicola*, showed a 5 to 8 % decrease in oviposition success when presented with sweet pepper leaves exposed to four hyperparasitoids (Fig. 4-1). However, *E. cerasicola* also generally had lower oviposition success across all hyperparasitoid doses compared to the other five primary parasitoid species but did exhibit reduced oviposition success when presented with leaves exposed to eight hyperparasitoids (Fig. 4-1). Primary parasitoid oviposition success was broadly comparable between the six species tested across all hyperparasitoid doses (generalised linear model with quasi-Poisson distribution: $F = 0.5151$; $d.f. = 4$, $P > 0.05$).

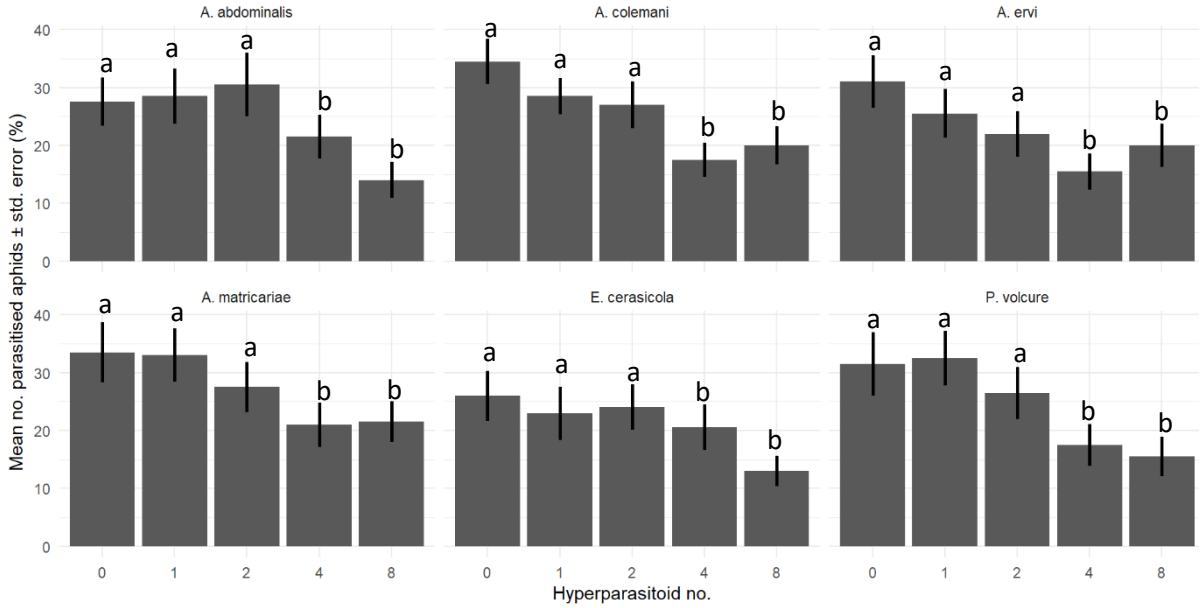


Figure 4-1 Percentage parasitism success (mean ± SE) of third instar *Myzus persicae* by six commercially available primary parasitoid species in response to *Asaphes suspensus* exposure on a sweet pepper (*Capsicum annum*) leaf (*N* = 20). Bars capped with different letters indicate statistically significant difference between hyperparasitoid dose ($P < 0.05$) based on Tukey's HSD method.

4.3.2 Effect of Hyperparasitised Mummy Exposure on Primary Parasitoid Oviposition Success

Hyperparasitised mummy presence had a negative impact on primary parasitoid oviposition success across all six species tested in no choice dose response experiments (generalised linear model with quasi-Poisson distribution: $F = 1.345$; *d.f.* = 4, $P < 0.001$) (Fig.4-2). The significant reduction in oviposition success was between one and two hyperparasitoid mummies. Primary parasitoid oviposition success was broadly comparable between the six species tested across all hyperparasitoid doses (generalised linear model with quasi-Poisson distribution: $F = 0.3564$; *d.f.* = 4, $P > 0.05$).

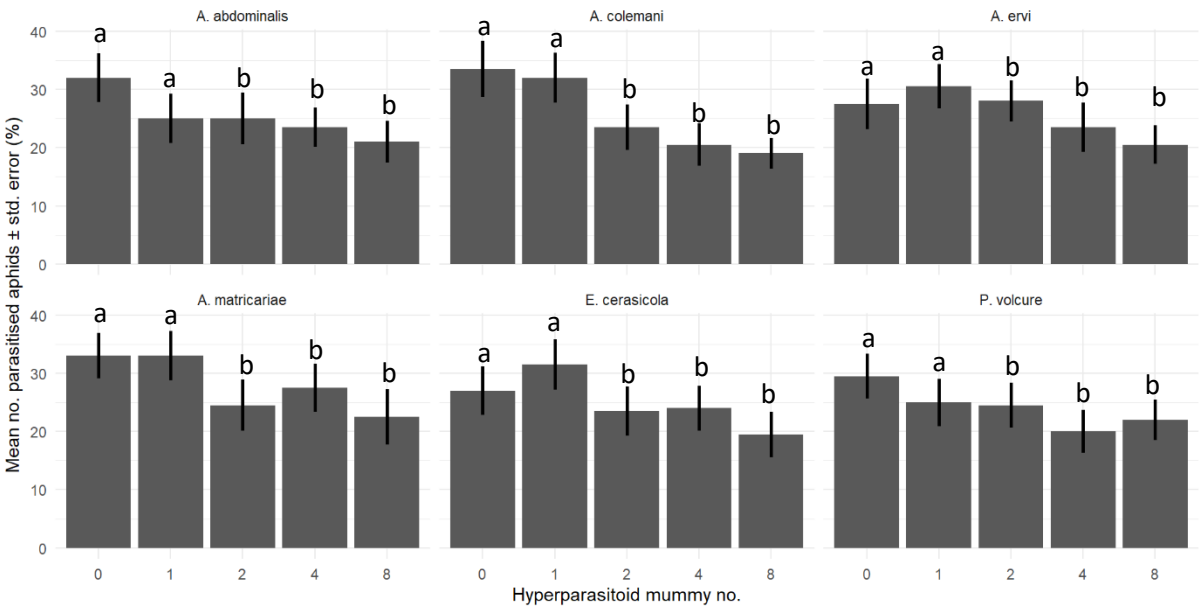


Figure 4-2 Percentage parasitism success (mean ± SE) of third instar *Myzus persicae* by six commercially available primary parasitoid species in response to *Asaphes suspensus* mummy exposure on a sweet pepper (*Capsicum annum*) leaf (*N* = 20). Bars capped with different letters indicate statistically significant difference between hyperparasitoid dose ($P < 0.05$) based on Tukey's HSD method.

4.3.3 Effect of Removing Adult Hyperparasitoid Trails on Primary Parasitoid Oviposition Success

Hyperparasitoid adult presence had no impact on primary parasitoid oviposition success across all six species tested when the leaves were washed following the hyperparasitoid exposure in no choice dose response experiments (generalised linear model with quasi-Poisson distribution: $F = 1.251$; *d.f.* = 4, $P > 0.05$) (Fig. 4-3). Primary parasitoid oviposition success was broadly comparable between the six species tested across all hyperparasitoid doses (generalised linear model with quasi-Poisson distribution: $F = 0.398$; *d.f.* = 4, $P > 0.05$).

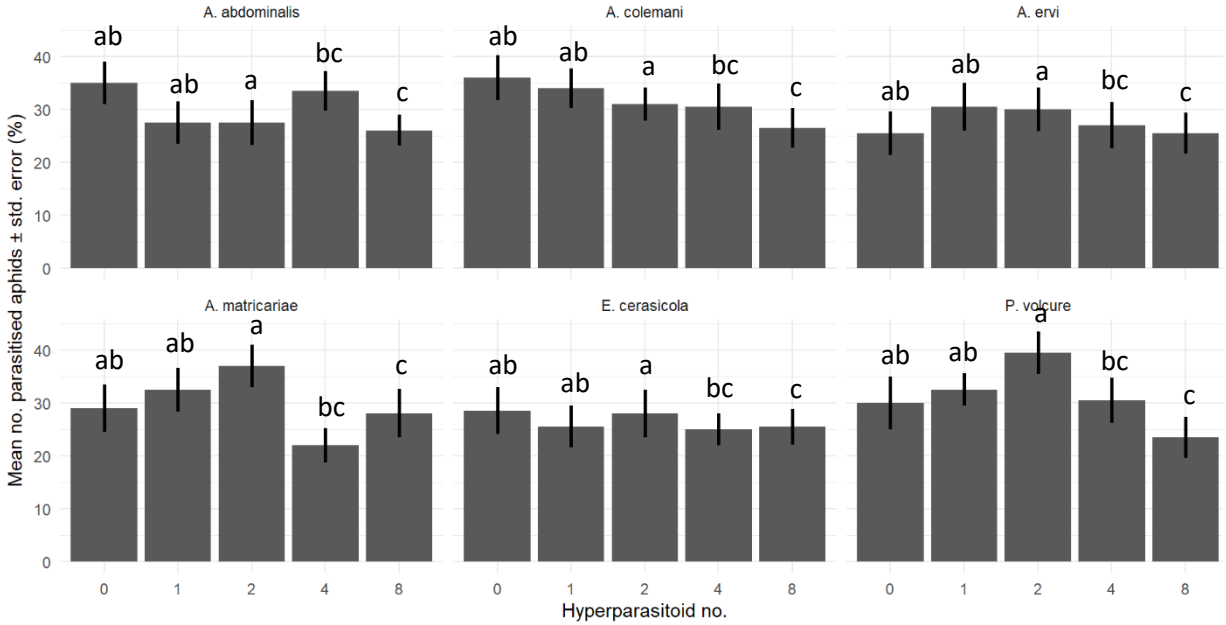


Figure 4-3 Percentage parasitism success (mean ± SE) of third instar *Myzus persicae* by six commercially available primary parasitoid species when leaves had been washed after *Asaphes suspensus* adults had been on a sweet pepper leaf (*Capsicum annum*) leaf (*N* = 20). Bars capped with different letters indicate statistically significant difference between hyperparasitoid dose (*P* >0.05) based on Tukey's HSD method.

4.3.4 Effect of Removing Hyperparasitoid Mummy Cues on Primary Parasitoids Oviposition Success

Hyperparasitoid adult presence had no impact on primary parasitoid oviposition success across all six species tested when the leaves were washed following the hyperparasitoid exposure in no choice dose response experiments (generalised linear model with quasi-Poisson distribution: $F = 0.931$; $d.f. = 4$, $P > 0.05$) (Fig. 4-4). Primary parasitoid oviposition success was broadly comparable between the six species tested across all hyperparasitoid doses (generalised linear model with quasi-Poisson distribution: $F = 0.141$; $d.f. = 4$, $P > 0.05$).

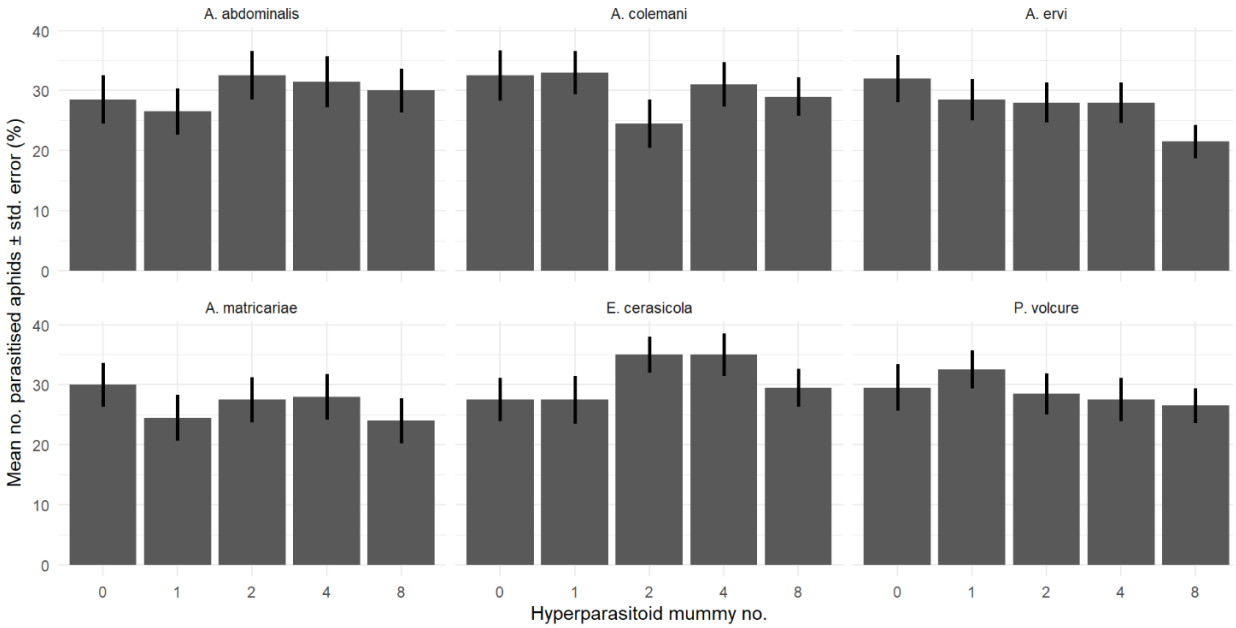


Figure 4-4 Percentage parasitism success (mean ± SE) of third instar *Myzus persicae* by six commercially available primary parasitoid species when sweet pepper leaf (*Capsicum annum*) and *Asaphes suspensus* mummies had been washed (*N* = 20).

4.3.5 Removal and Re-Application of Anti-Oviposition Cues Associated with Adult Hyperparasitoid Exposure

Hyperparasitoid adult presence had no impact on primary parasitoid oviposition success across all four species tested when the leaves were washed following the hyperparasitoid exposure and the solution reapplied to fresh pepper leaves (*Capsicum annum*) in no choice dose response experiments (generalised linear model with quasi-Poisson distribution: $F = 0.921$; $d.f. = 2$, $P > 0.05$) (Fig. 4-5). Primary parasitoid oviposition success was comparable between the four species tested across all hyperparasitoid doses (generalised linear model with quasi-Poisson distribution: $F = 0.398$; $d.f. = 3$ $P > 0.05$).

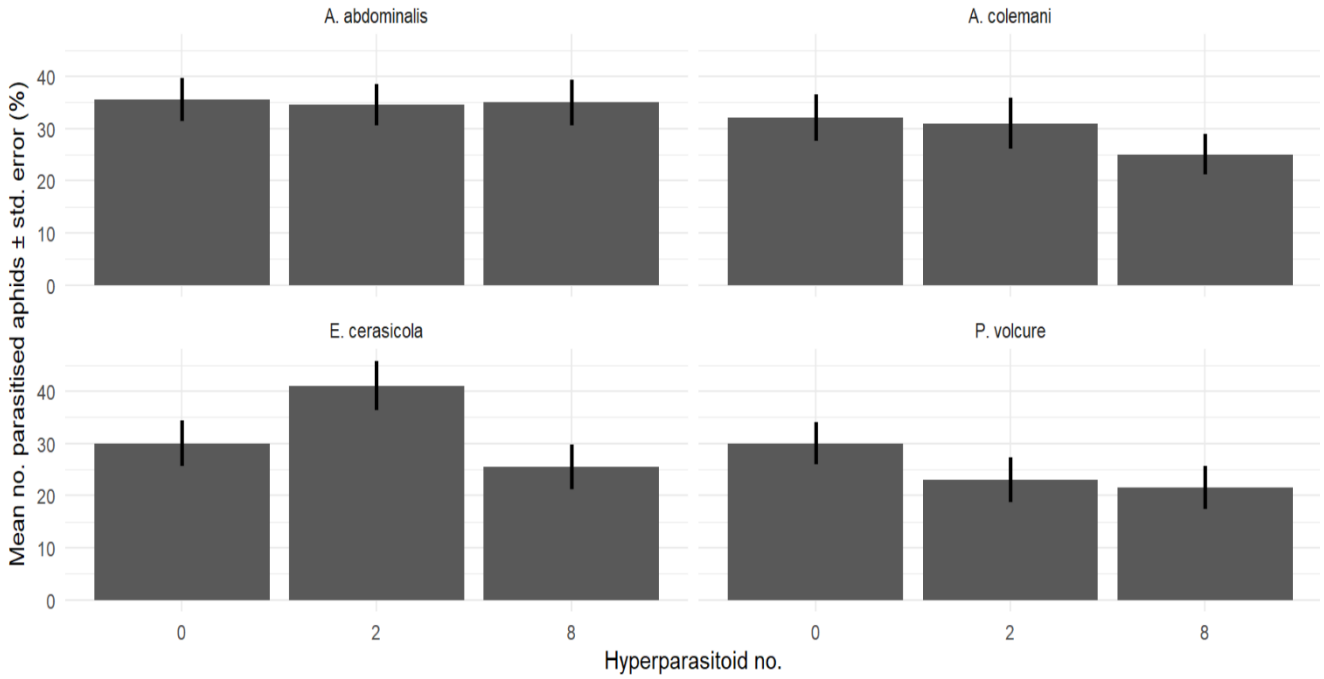


Figure 4-5 Percentage parasitism success (mean ± SE) of third instar *Myzus persicae* by six commercially available primary parasitoid species in response to adult hyperparasitoid cues being applied to a sweet pepper leaf (*Capsicum annum*) (*N* = 20).

4.3.6 Removal and Re-Application of Anti-Oviposition Cues Associated with Adult Hyperparasitoid Exposure

Hyperparasitised mummy presence had no impact on primary parasitoid oviposition success across all four species tested when the leaves and mummies were washed and the solution reapplied to fresh pepper leaves (*Capsicum annum*) in no choice dose response experiments (generalised linear model with quasi-Poisson distribution: $F = 1.132$; $d.f. = 2$, $P > 0.05$) (Fig. 4.6). Primary parasitoid oviposition success was broadly comparable between the four species tested across all hyperparasitoid doses (generalised linear model with quasi-Poisson distribution: $F = 0.392$; $d.f. = 3$, $P > 0.05$).

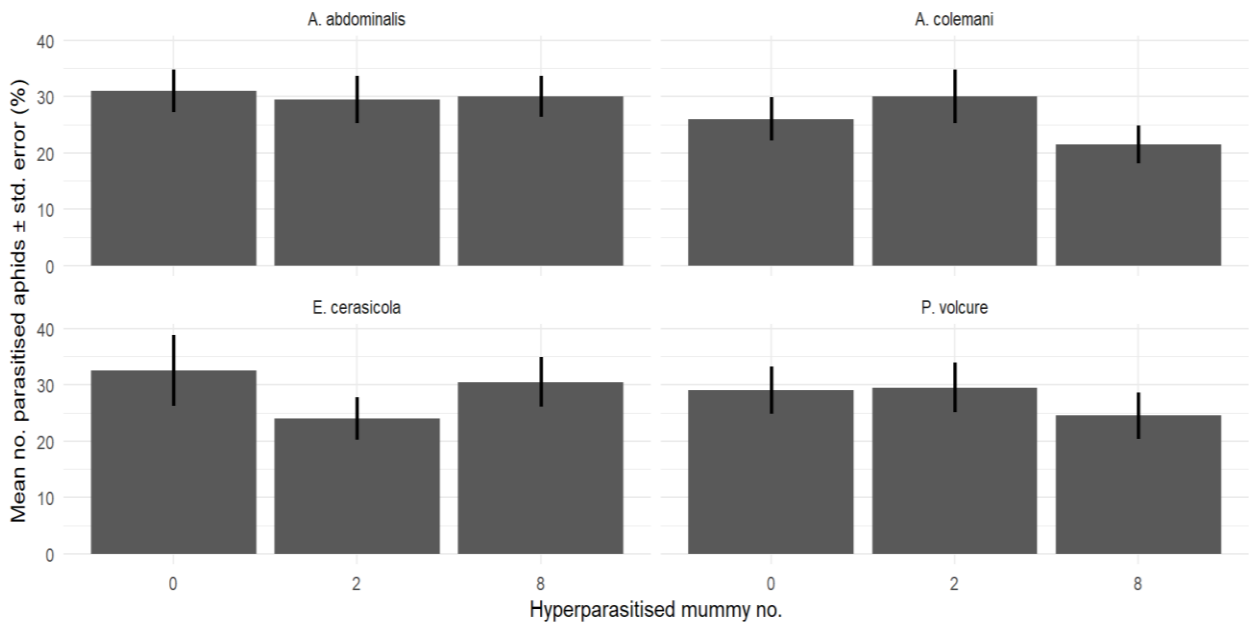


Figure 4.6 Percentage parasitism success (mean ± SE) of third instar *Myzus persicae* by six commercially available primary parasitoid species in response to hyperparasitised mummy cues being applied to a sweet pepper leaf (*Capsicum annum*) (*N* = 20).

4.4 Discussion

A. colemani, A. ervi, A. matricariae, A. abdominalis, E. cerasicola and *P. volucre* are six commercially available species of hymenopteran primary parasitoid available to UK growers for aphid control (van Lenteren, 2018). This study aimed to establish whether plant exposure to hyperparasitoids prior to primary parasitoid host-seeking impacts oviposition success under laboratory conditions. This will provide better understanding of hyperparasitoid ecology and inform their management in protected horticulture crops.

All primary parasitoid are less likely to oviposit in *M. persicae* on leaves where four or eight *A. suspensus* adults have been present, though this behaviour was only observed after exposure to eight adults in *Ephedrus cerasicola*. The reduced parasitism was only observed when hyperparasitoid adults were present. These findings suggest that primary parasitoids are unable to detect the presence of one or two hyperparasitoids on an aphid-infested plant or that they are not deterred from parasitising aphids in areas exposed to low numbers of hyperparasitoids. Parallels can be drawn between this study and aphid-predator and predator-predator interactions. Oviposition avoidance behaviour has previously been observed in *A. ervi* when presented with leaves visited by the predatory seven-spot lady bird, *Coccinella septempunctata* L. (Coleoptera: Coccineliidae), within the preceding 24 hours (Nakashima

et al., 2004). Our study exposed parasitoids to leaves previously exposed to adult hyperparasitoids for one hour, but the study conducted by Nakashima *et al.* (2004) suggests that semiochemicals emitted by *C. septempunctata* remain on the leaf causing avoidance behaviour for up to 24 hours. There is, therefore, a possibility that chemical cues potentially deposited by predators and hyperparasitoids may be biologically active for extended periods of time, depending on environmental conditions.

Overall, the results show that fewer aphids are parasitised on leaves previously visited by four or eight hyperparasitoids, compared with one or two. Additionally, *A. abdominalis, A. colemani, A. ervi* and *A. matricariae* have a similar trend showing that zero to two hyperparasitised mummies do not have a large effect in primary parasitoid oviposition success but there is a decrease when there are four or eight hyperparasitised mummies, with an overall decline. *E. cerasicola*, on the other hand, shows a greater decrease in number of aphids parasitised with only two and four hyperparasitised mummies present and a further decrease when there are eight hyperparasitised mummies present. *P. volucre* demonstrated a decrease in oviposition success when there was one or two hyperparasitised mummies present compared with none and a further decrease when there were four or eight hyperparasitised mummies. The overall effect observed, that primary parasitoids are less likely to oviposit in areas of high hyperparasitoid density, supports UK grower experience where they state that primary parasitoid efficacy is reduced by exposure to hyperparasitoids (Ridley *et al.*, 2019).

The hyperparasitoid-parasitoid interaction studied here investigates how hyperparasitoid presence within a crop negatively impacts primary parasitoid oviposition. Contrastingly the predator-predator effect shown in a study by Fonseca *et al.,* using two predatory mite species *Phytoseiulus macropilis* (Banks) and *Neoseiulus californicus* (McGregor), two species used in the control of the pest species the two-spotted spider mite (*Tetranychus urticae* Koch), shows these predators do not avoid plants on which the other species is present and that their oviposition success in not affected (Fonseca et al., 2020). However, predator suppression has been demonstrated between predatory mites *Typhlodrmalus manihoti* (Moraes) and *Euseius fustis* (Pritchard & Baker) used in the control of the cassava green mite pest (*Mononychellus tanajoa* Bondar) where *E. fustis* was shown to eliminate *T. manihotti* populations on cassava crops (*Manihot esculenta* Crantz). This suggests that the affect that predators and parasitoids have on one another varies between study systems and, therefore, further study on parasitoid-hyperparasitoid interactions is needed.

Our study suggests that where one or two hyperparasitoids have previously been present on a leaf, primary parasitoids continue to reproduce in the area which corroborates with research conducted showing that *A. suspensus* does not eliminate entire populations of *A. ervi* in cropping systems (Schooler *et al.*, 2011). This study suggests that being exposed to

hyperparasitoids may not eliminate an entire parasitoid population or prevent them for reproducing but may reduce it where present in high numbers similarly to a study by Höller *et al*. (1993) showing that fewer aphids are parasitised in areas of high hyperparasitoid density using 14 species of aphid primary parasitoids and 18 species of aphid hyperparasitoids.

The effect that *A. suspensus* exposure was shown to have on primary parasitoid oviposition success is hypothesised to be a result of hyperparasitoid-derived semiochemical cues as the wash experiments indicate that primary parasitoid oviposition success is higher on washed leaves where there has previously been high hyperparasitoid pressure. This is particularly apparent in the experiment where the leaves and mummies were washed as there would still be the visual cues of the aphid mummies, yet this did not deter the primary parasitoids from ovipositing in that area as strongly as when the leaves and mummies were not washed. Future studies investigating washed and unwashed leaves/leaves and mummies together to enable a comparative analysis would enable further understanding of whether primary parasitoids do appear to avoid ovipositing in high numbers in areas where hyperparasitoids are present but not that they do not oviposit at all.

Aphid honeydew acts as a high energy source and foraging cue for primary parasitoids, and that, in response to high hyperparasitoid numbers, primary parasitoids oviposit in aphid hosts over several areas within cropping systems in order to reduce the risk of offspring mortality (Mackauer and Völkl, 1993). Though there is yet to be a study investigating how semiochemicals originating from *A. suspensus* may impact the foraging behaviour of these primary parasitoid species, at this stage one could speculate that the mechanistic justification is that it is beneficial to the hyperparasitoid species for parasitoids to avoid ovipositing in areas that the hyperparasitoid has already visited as the hyperparasitoid may not return to hyperparasitise the mummified aphid. Instead, if the parasitoid finds an aphid host elsewhere perhaps there's a higher likelihood that the hyperparasitoid would find that host. Höller *et al.* (1991) show that oviposition-deterring semiochemicals are used by *Dendrocerus carpenteri* (Curtis) to prevent multiparasitism (when two or more parasitoid species parasitise the same host) or superparasitism (when two or more parasitoids of the same species parasitise the same host). Though Höller *et al*. (1991) studied a different interaction, it does demonstrate the presence of oviposition-deterrent pheromones in hymenopteran hyperparasitoids, suggesting oviposition-semiochemicals may also be used by hyperparasitoids to manipulate primary parasitoid host selection within a crop (Alphen and Visser, 1990; Fisher, 1961).

The experiment investigating whether the water solution used to wash leaves after hyperparasitoids have been on them could then be applied to another pepper leaf further strengthens the justification for interpreting the primary parasitoid deterrence as being a response to hyperparasitoid semiochemicals. The results showed no significance in primary parasitoid oviposition success relative to hyperparasitoid presence at any number. However,

there may still be potential to develop a methodology of effectively collecting and reapplying chemical cues to leaves to reduce primary parasitoid oviposition. For instance, distilled water was used in these experiments, much like a 1996 study on the effect of aphid semiochemicals on *A. ervi* foraging behaviour (Du et al., 1996). However there have been experiments conducted that use solvents such as ethanol, for example Nakashima *et al*., (2004), used ethanol to remove ladybird (*Coccinella septempunctata*) semiochemicals, which was also then effectively used to reapply the semiochemical solution to leaves to attract *A. ervi* (Nakashima et al., 2004). Further investigation into more effective solvents potentially used to remove and reapply chemical cues would help move the research forward, followed by more specific analysis of semiochemicals that have caused the effects shown in this data should be carried out to identify specific chemicals that the primary parasitoids may be detecting and using to inform their oviposition decisions. Further research into the chemical cues that *A. suspensus* detects from the primary parasitoids would also be integral to developing hyperparasitoid control strategies such as the push-pull strategy where the hyperparasitoid is pushed from their parasitoid host and simultaneously pulled into a chemically-baited trap (Cusumano et al., 2020). To do this, however, information and understanding is needed about the semiochemicals used by hyperparasitoids in locating parasitised hosts. Further research should focus on determining the exact source of semiochemicals that limits primary parasitoid oviposition success.

Though the statistical analysis shows no significant difference between species in all six experiments, oviposition success in *E. cerasicola* does appear to have a different pattern to the other species after adult presence on the unwashed leaves and on leaves that have the distilled water painted on after being used to clean leaves after adult presence. These patterns suggest that, though the same affect is shown in response to hyperparasitoid presence, *E. cerasicola* may have a higher threshold than the other primary parasitoid species. Of the eggs that were laid inside of the aphids during the experiments by the primary parasitoids, the number of hyperparasitoids present on the leaf did not show an effect on the sex ratio or development of the parasitoids. There was no significant difference between the sizes (weight, body length, head width, hind leg length or antenna length) of the wasps that emerged, suggesting that, though fewer aphids are selected as hosts when occupying leaves where hyperparasitoids were previously present, those that are selected do not affect the development of the primary parasitoid in any of the ways observed in this study.

Using all six of the commercially available primary parasitoid species in the UK and *Asaphes suspensus,* one of the most commonly found hyperparasitoids in protected sweet pepper crops in the UK (de Boer et al., 2019; van Lenteren et al., 2018) the results show that the primary parasitoids' oviposition success is negatively affected by hyperparasitoid exposure when four or eight hyperparasitoids have been present on a single leaf. The information

produced by this research can be built upon for further research into the specific semiochemicals causing the effects shown in this study and into the semiochemicals that hyperparasitoids use to detect and locate primary parasitoid hosts. All of which can lead towards developing effective hyperparasitoid control strategies in protected sweet pepper cropping systems in the UK.

CHAPTER 5

GENERAL DISCUSSION

This project aimed to develop a more in-depth understanding of hymenopteran hyperparasitoids as the fourth trophic level that they occupy is comparatively understudied in agri-ecosystems. Hyperparasitoids threaten pest management programmes in cropping systems that utilise hymenopteran primary parasitoids to control aphids such as peach potato aphid, one of the most economically important aphid pests, by using the primary parasitoids as their hosts (Sullivan, 1987). With more information on primary parasitoid foraging behaviour and how hyperparasitoids may influence this, development of effective hyperparasitoid management/control methods in UK cropping systems could be developed (Cusumano et al., 2020).

Initial experiments carried out in Chapter Three focussed on studying aspects of fundamental ecology between the hyperparasitoid *Asaphes suspensus*, primary parasitoids and *M. persicae* that are found in UK sweet pepper (*Capsicum* annum) crops. These experiments focussed on peach potato aphid (*Myzus persicae*) and six commercially available primary parasitoid species: *A. colemani, A. ervi, A. matricariae, A. abdominalis, E. cerasicola* and *P. volucre.* Total development time of these species was observed from oviposition through to adult emergence or, in the case of peach potato aphid, from larviposition to reproductive adult. Peach potato aphids took a mean time to eight days to reach reproductive adulthood from first instar nymphs and this corroborates previous research (Blackman, 2009). The three *Aphidius* species took a mean of 13 to 14 days to develop from the point of oviposition to adult emergence, *E. cerasicola* took a mean time of just between 14 and 15 days, *P. volucre* took the longest time with a mean of 17.4 days. *Asaphes suspensus* took between 17 and 19 days to fully develop from oviposition to emerging adult which was not significantly affected by which of the six parasitoid species it used as its host. These observations corroborate recent research (de Boer et al., 2019; Henry et al., 2005; Perdikis et al., 2004; Sigsgaard, 2000).

The apparent preference of primary parasitoids for specific aphid host developmental stage was investigated using choice and no-choice experiments offering individual parasitoid species peach potato aphids of different ages. Primary parasitoids, such as *A. colemani*, are thought to display a preference for the intermediate (third and frouth) instar stages (Sampaio et al., 2008). This study validates this previously reported information as *A. colemani* showed higher parasitism levels in third instar peach potato aphids over all other life stages when given no choice between aphid developmental stage but also selected third instar aphids the most when given a choice of aphids at different developmental stages. Experiments carried out in Chapter Four investigated the impact of hyperparasitoid presence on a host plant has on primary parasitoid foraging behaviour. These experiments specifically focussed on the hyperparasitoid semiochemical trails deposited on the plant and whether primary parasitoids were able to detect these once the hyperparasitoid had left the plant. This experiment demonstrated that primary parasitoids parasitised fewer aphids when there had been four or eight hyperparasitoids previously present on the leaf, likely suggesting hyperparasitoids deposit a non-volatile semiochemical cue that the primary parasitoid detects and that might affect their oviposition success.

Research completed in this study has helped develop knowledge on hyperparasitoid ecology and the wider trophic system that they occupy. The development study on the six commercially available primary parasitoid species showed that though there were differences in time, the overall development time was similar and fell within a three-week window. It also showed that no difference in the hyperparasitoid's development on the six different host species. The results of this part of the study suggest that growers can swap between primary parasitoid species without having any effect on hyperarasitoid development.

With the evidence of hyperparasitoid semiochemical cues deterring primary parasitoids from the leaf, larger scale in-field studies could be carried out to investigate whether this affect is seen in cropping systems to confirm grower experiences that have been reported (Fray et al., 2015). Further studies investigating the specific semiochemicals causing the effects shown in this hyperparasitoid trail study using headspace sampling and GC-MS would be invaluable to the development of hyperparasitoid control strategies that will benefit IPM strategies that utilise hymenopteran primary parasitoids. A horticultural practice of washing crops following the detection of hyperparasitoids with an effective solution may be developed following further research into washing off anti-oviposition cues as investigated in this project. Understanding primary parasitoid-hyperparasitoid interactions could also be expanded upon by investigating the semiochemicals that hyperparasitoids might use to detect and locate primary parasitoid hosts as with Cusumano *et al*'s research on hyperparasitoids using herbivore induce plant volatiles to locate potential hosts (Cusumano et al., 2020).

This project explored and met its research aims to improve understanding of the fundamental ecology of the aphid, the primary parasitoids and the hyperparasitoid trophic system by observing their development as well as primary parasitoid host age preferences. It also explored how the presence of hyperparasitoids presence amongst the crop plant might affect the primary parasitoids' host selection. This has provided a foundation for hyperparasitoid research to develop the chemical ecology of hyperparasitoids and their use of semiochemicals in host selection. Further information on this could be used in developing hyperparasitoid monitoring and control techniques in the UK's protected sweet pepper crops.

Supplementary information:

Table S1 Measurements of the primary parasitoids that emerged from the aphids that were parasitised during the experiment using unwashed leaves that have previously had adult hyperparasitoids on, showing the mean weight, body length, head width, hind leg length and antenna length (standard error inclusive) (*N* = 20).

Species	Hyper-parasitoid number	Mean weight (µg ± SE)	Mean body length (mm ± SE)	Mean head width (mm ± SE)	Mean leg length (mm ± SE)	Mean antenna length (mm ± SE)
A. colemani	0	69 ± 3.24E-06	2.58 ± 0.051	0.52 ± 0.019	2.2130 ± 0.044	1.635 ± 0.048
	1	74 ± 3.12E-06	2.47 ± 0.060	0.52 ± 0.027	2.0710 ± 0.029	1.711 ± 0.029
	2	81 ± 3.04E-06	2.50 ± 0.026	0.53 ± 0.023	2.1255 ± 0.017	1.837 ± 0.024
	4	68 ± 3.85E-06	2.33 ± 0.045	0.50 ± 0.019	2.0025 ± 0.041	1.641 ± 0.039
	8	69 ± 4.51E-06	2.38 ± 0.026	0.52 ± 0.016	2.0485 ± 0.021	1.705 ± 0.035
A. ervi	0	102 ± 1.97E-06	3.54 ± 0.049	0.43 ± 0.011	2.5437 ± 0.037	1.808 ± 0.029
	1	103 ± 1.79E-06	3.58 ± 0.044	0.44 ± 0.019	2.4830 ± 0.048	1.764 ± 0.050
	2	105 ± 1.77E-06	3.66 ± 0.035	0.42 ± 0.014	2.5425 ± 0.028	1.812 ± 0.024
	4	103 ± 2.09E-06	3.69 ± 0.038	0.46 ± 0.020	2.4685 ± 0.026	1.820 ± 0.022
	8	98 ± 5.04E-06	3.69 ± 0.036	0.42 ± 0.019	2.5570 ± 0.024	1.872 ± 0.021
A. matricariae	0	71 ± 1.84E-06	2.02 ± 0.022	0.39 ± 0.010	1.9025 ± 0.023	1.359 ± 0.041
	1	71 ± 1.78E-06	1.91 ± 0.035	0.39 ± 0.018	1.8285 ± 0.028	1.418 ± 0.024
	2	70 ± 1.60E-06	1.90 ± 0.032	0.43 ± 0.016	1.7835 ± 0.028	1.540 ± 0.032
	4	71 ± 2.03E-06	1.75 ± 0.043	0.38 ± 0.016	1.5695 ± 0.039	1.484 ± 0.040
	8	75 ± 2.24E-06	1.95 ± 0.021	0.40 ± 0.013	1.7925 ± 0.024	1.469 ± 0.046
A. abdominalis	0	78 ± 5.07E-06	0.000 ± 1.389	0.022 ± 0.421	1.1463 ± 0.053	0.322 ± 0.016
	1	74 ± 3.88E-06	1.00 ± 1.383	0.021 ± 0.422	0.9685 ± 0.033	0.330 ± 0.016
	2	77 ± 3.87E-06	2.00 ± 1.349	0.017 ± 0.443	1.1485 ± 0.023	0.400 ± 0.012
	4	72 ± 4.02E-06	4.00 ± 1.453	0.043 ± 0.416	1.2280 ±0.031	0.396 ± 0.022
	8	78 ± 3.94E-06	8.00 ± 1.338	0.016 ± 0.440	0.9825 ± 0.019	0.387 ± 0.020
E. cerasicola	0	83 ± 3.90E-06	1.70 ± 0.020	0.52 ± 0.014	1.5168 ± 0.012	1.342 ± 0.020
	1	81 ± 3.47E-06	1.72 ± 0.028	0.53 ± 0.017	1.5000 ± 0.025	1.361 ± 0.010
	2	78 ± 3.26E-06	1.77 ± 0.024	0.52 ± 0.011	1.4985 ± 0.021	1.319 ± 0.019
	4	83 ± .49E-06	1.76 ± 0.019	0.54 ± 0.014	1.496 ± 0.015	1.310 ± 0.020
	8	71 ±3.80E-06	1.77 ± 0.013	0.52 ± 0.013	1.4710 ± 0.014	1.298 ± 0.019
P. volucre	0	70 ± 2.79E-06	2.02 ± 0.022	0.39 ± 0.010	1.8818 ± 0.029	1.402 ± 0.036
	1	71 ± 2.01E-06	1.91 ± 0.035	0.40 ± 0.020	1.8210 ± 0.028	1.422 ± 0.030
	2	72 ± 1.66E-06	1.90 ± 0.032	0.44 ± 0.017	1.7895 ± 0.029	1.538 ± 0.032

4	71 ± 2.48E-06	1.75 ± 0.043	0.40 ± 0.019	1.5745± 0.037	1.491 ± 0.042
8	76 ± 2.08E-06	1.95 ± 0.021	0.40 ± 0.015	1.8025 ± 0.0294	1.448 ± 0.044

Table S2 Percentage of female primary parasitoids that emerged from the aphids that were parasitised during the experiment using unwashed leaves after adult hyperparasitoid exposure (*N = 20*).

Hyper-parasitoid No.	Percentage female of emerged primary parasitoids					
	A. colemani	***A. ervi***	***A. matricariae***	***A. abdominalis***	***E. cerasicola***	***P. volucre***
0	46.4	54.8	49.3	50.9	51.9	47.6
1	45.6	49.0	53.0	50.9	45.7	50.8
2	51.9	47.7	52.7	54.1	52.1	45.3
4	48.2	54.8	45.2	44.2	46.3	45.7
8	51.4	54.8	51.2	50.0	50.0	45.2

Table S3 The measurements of the primary parasitoids that emerged from the aphids that were parasitised during the experiment using unwashed leaves with hyperparasitised mummies on, showing the mean weight, body length, head width, hind leg length and antenna length (standard error inclusive) (*N* = 20).

Species	Hyper-parasit-oid number	Mean weight (µg ± SE)	Mean body length (mm± SE)	Mean head width (mm± SE)	Mean leg length (mm± SE)	Mean antenna length (mm± SE)
A. colemani	0	91 ±3.2 E-06	2.583 ±0.052	0.519 ±0.019	2.213 ±0.045	1.6346 ±0.048
	1	69 ±3.3 E-06	2.471 ±0.06	0.523 ±0.027	2.071 ±0.030	1.7105 ±0.030
	2	80 ±3.0 E-06	2.500 ±0.025	0.529 ±0.023	2.125 ±0.017	1.8370 ±0.024
	4	67 ±3.8 E-06	2.333 ±0.045	0.501 ±0.018	2.002 ±0.041	1.6410 ±0.040
	8	69 ±4.5 E-06	2.384 ±0.026	0.521 ±0.016	2.048 ±0.021	1.7050 ±0.035
A. ervi	0	102 ±2.0 E-06	3.538 ±0.048	0.429 ±0.011	2.543 ±0.38	1.8078 ±0.030
	1	103 ±1.8 E-06	3.580 ±0.044	0.439 ±0.019	2.483 ±0.048	1.7635 ±0.050
	2	10 5±1.8 E-06	3.654 ±0.035	0.419 ±0.014	2.545 ±0.028	1.8115 ±0.024
	4	103 ±2.1 E-06	3.687 ±0.037	0.461 ±0.020	2.468 ±0.026	1.8200 ±0.022
	8	99 ±5.0 E-06	3.685 ±0.036	0.416 ±0.019	2.557 ±0.024	1.8720 ±0.021
A. matricariae	0	71 ±1.8 E-06	2.016 ±0.021	0.393 ±0.010	1.902 ±0.023	1.3591 ±0.041
	1	71 ±1.8 E-06	1.906 ±0.034	0.388 ±0.018	1.828 ±0.025	1.4180 ±0.024
	2	70 ±1.6 E-06	1.899 ±0.032	0.432 ±0.016	1.783 ±0.025	1.5400 ±0.032
	4	71 ±2.0 E-06	1.751 ±0.043	0.384 ±0.016	1.569 ±0.039	1.4840 ±0.040
	8	74 ±2.2 E-06	1.948 ±0.021	0.397 ±0.013	1.793 ±0.024	1.4690 ±0.0453
A. abdominalis	0	78 ±5.1 E-06	1.388 ±0.022	0.421 ±0.015	1.146 ±0.053	0.3215 ±0.016
	1	73 ±3.9 E-06	1.385 ±0.021	0.422 ±0.014	0.969 ±0.033	0.3300 ±0.016
	2	77 ±3.9 E-06	1.348 ±0.017	0.443 ±0.016	1.148 ±0.023	0.4000 ±0.011
	4	71 ±3.4 E- 06	1.453 ±0.042	0.415 ±0.021	1.228 ±0.031	0.3960 ±0.022

	8	77 ±3.9 E-06	1.338 ±0.016	0.440 ±0.020	0.983 ±0.019	0.3870 ±0.020
E. cerasicola	0	83 ±3.9 E-06	1.740 ±0.019	0.519 ±0.014	1.517 ±0.012	1.3420 ±0.020
	1	81 ±3.5 E-06	1.715 ±0.028	0.525 ±0.017	1.500 ±0.025	1.3605 ±0.019
	2	87 ±3.3 E-06	1.773 ±0.024	0.520 ±0.011	1.499 ±0.021	1.3185 ±0.019
	4	82 ±3.5 E-06	1.760 ±0.018	0.538 ±0.014	1.49 ±0.015	1.3104 ±0.021
	8	74 ±3.8 E-06	1.765 ±0.013	0.523 ±0.013	1.471 ±0.038	1.2975 ±0.020
P. volucre	0	70 ±2.8 E-06	2.016 ±0.021	0.392 ±0.010	1.882 ±0.029	1.4021 ±0.036
	1	71 ±2.0 E-06	1.906 ±0.034	0.399 ±0.020	1.821 ±0.028	1.4215 ±0.030
	2	71 ±1.7 E-06	1.899 ±0.032	0.437 ±0.017	1.789 ±0.028	1.5375 ±0.032
	4	70 ±2.5 E-06	1.751 ±0.043	0.398 ±0.019	1.575 ±0.037	1.4910 ±0.042
	8	75 ±2.1 E-06	1.948 ±0.021	0.398 ±0.015	1.803 ±0.029	1.4480 ±0.044

Table S4 The percentage of female primary parasitoids that emerged from the aphids that were parasitised during the experiment using unwashed leaves with hyperparasitised mummies present (*N = 20*).

	Percentage female of emerged primary parasitoids						
	A. colemani	***A. ervi***	***A. matricariae***	***A. abdominalis***	***E. cerasicola***	***P. volucre***	**Hyper-parasitoid No.**
0	45.4	54.8	49.3	50.9	51.9	47.6	
1	47.3	53.0	52.4	54.1	45.7	45.2	
2	52.2	47.7	52.7	54.8	52.1	50.0	
4	49.4	54.8	45.2	54.8	46.3	46.3	
8	54.1	54.8	51.2	50.0	54.8	45.2	

Table S5 The measurements of the primary parasitoids that emerged from the aphids that were parasitised during the experiment using washed leaves that had previously had hyperparasitised adults on, showing the mean weight, body length, head width, hind leg length and antenna length (standard error inclusive) (*N* = 20).

Species	Hyper-parasit-oid number	Mean weight (µg ± SE)	Mean body length (mm)	Mean head width (mm)	Mean leg length (mm)	Mean antenna length (mm)
A. colemani	0	6.9 ±3.2 E-06	2.58 ±0.051	0.52 ±0.019	2.213 ±0.045	1.635 ±0.048
	1	6.9 ±3.3 E-06	2.47 ±0.06	0.52 ±0.027	2.071 ±0.030	1.711 ±0.030
	2	8.0 ±3.0 E-06	2.50 ±0.026	0.53 ±0.023	2.126 ±0.017	1.837 ±0.024
	4	6.8 ±3.8 E-06	2.33 ±0.045	0.50 ±0.018	2.003 ±0.041	1.641 ±0.040
	8	7.0 ±4.5 E-06	2.38 ±0.026	0.52 ±0.016	2.049 ±0.021	1.705 ±0.035
A. ervi	0	10.2 ±2.0 E-06	3.54 ±0.049	0.43 ±0.011	2.544 ±0.038	1.808 ±0.029
	1	10.4 ±1.8 E-06	3.58 ±0.044	0.44 ±0.019	2.483 ±0.048	1.764 ±0.050
	2	10.6 ±1.8 E-06	3.66 ±0.038	0.42 ±0.014	2.543 ±0.028	1.812 ±0.023
	4	10.4 ±2.1 E-06	3.69 ±0.035	0.46 ±0.020	2.469 ±0.026	1.820 ±0.022
	8	10.0 ±0.5 E-06	3.69 ±0.036	0.41 ±0.019	2.557 ±0.024	1.872 ±0.021
A. matricariae	0	7.1 ±1.8 E-06	2.02 ±0.022	0.39 ±0.010	1.903 ±0.026	1.359 ±0.041
	1	7.1 ±1.6 E-06	1.91 ±0.035	0.39 ±0.018	1.829 ±0.028	1.418 ±0.024
	2	7.0 ±1.6 E-06	1.89 ±0.032	0.43 ±0.016	1.784 ±0.028	0.032 ±0.040
	4	7.1 ±2.0 E-06	1.75 ±0.043	0.38 ±0.016	1.570 ±0.39	1.484 ±0.040
	8	7.5 ±2.2 E-06	1.95 ±0.021	0.40 ±0.013	1.793 ±0.024	1.469 ±0.046
A. abdominalis	0	7.8 ±5.1 E-06	1.39 ±0.022	0.42 ±0.015	1.146 ±0.053	0.322 ±0.016
	1	7.4 ±3.9 E-06	1.38 ±0.021	0.42 ±0.014	0.969 ±0.033	0.330 ±0.016
	2	7.7 ±3.9 E-06	1.35 ±0.017	0.44 ±0.013	1.149 ±0.023	0.400 ±0.012
	4	7.2 ±3.9 E-06	1.45 ±0.042	0.41 ±0.012	1.228 ±0.031	0.396 ±0.022
	8	7.8 ±3.9 E-06	1.34 ±0.016	0.44 ±0.016	0.983 ±0.019	0.387 ±0.020
E. cerasicola	0	8.3 ±39 E-06	1.74 ±0.019	0.52 ±0.014	1.517 ±0.012	1.342 ±0.020
	1	8.1 ±3.5 E-06	0.03 ±0.019	0.53 ±0.017	1.500 ±0.025	1.361 ±0.020
	2	8.8 ±3.3 E-06	1.78 ±0.024	0.52 ±0.011	1.499 ±0.021	1.319 ±0.019
	4	8.3 ±3.5 E-06	1.76 ±0.018	0.54 ±0.014	1.496 ±0.015	1.310 ±0.020
	8	7.4 ±3.8 E-06	1.77 ±0.013	0.52 ±0.013	1.471 ±0.014	1.298 ±0.020
P. volucre	0	7.0 ±2.8 E-06	2.01 ±0.021	0.39 ±0.010	1.882 ±0.029	1.402 ±0.036
	1	7.2 ±2.0 E-06	1.91 ±0.035	0.39 ±0.020	1.789 ±0.037	1.422 ±0.030
	2	7.0 ±2.0 E-06	1.89 ±0.032	0.47 ±0.020	1.654 ±0.041	1.538 ±0.032
	4	7.0 ±2.5 E-06	1.75 ±0.043	0.39 ±0.019	1.575 ±0.037	1.491 ±0.042
	8	7.5 ±2.1 E-06	1.95 ±0.021	0.39 ±0.015	1.803 ±0.029	1.460 ±0.041

Table S6 The percentage of female primary parasitoids that emerged from the aphids that were parasitised during the experiment using washed leaves after adult hyperparasitoid exposure (*N = 20*).

	Percentage female of emerged primary parasitoids						
	A. colemani	***A. ervi***	***A. matricariae***	***A. abdominalis***	***E. cerasicola***	***P. volucre***	**Hyperparasitoid No.**
0	45.4	54.8	49.3	50.9	51.9	47.6	
1	53.3	53.0	54.3	51.0	45.7	45.2	
2	52.2	49.8	52.7	54.1	52.1	45.2	
4	54.1	54.8	53.0	46.3	46.3	46.3	
8	54.1	45.4	51.2	52.7	48.9	51.9	

Table S7 The measurements of the primary parasitoids that emerged from the aphids that were parasitised during the experiment using washed leaves with hyperparasitised mummies present showing the mean weight, body length, head width, hind leg length and antenna length (standard error inclusive) (*N* = 20).

Species	Hyper-parasitoid number	Mean weight (µg ± SE)	Mean body length (mm)	Mean head width (mm)	Mean leg length (mm)	Mean antenna length (mm)
A. colemani	0	6.9 ±3.2E-06	2.58 ±0.051	0.51 ±0.019	2.213 ±0.045	1.634 ±0.048
	1	6.9 ±3.3E-06	2.47 ±0.060	0.52 ±0.027	2.071 ±0.029	1.710 ±0.030
	2	8.0 ±3.0E-06	2.50 ±0.025	0.52 ±0.023	2.125 ±0.017	1.837 ±.0.024
	4	6.8 ±3.8E-06	2.33 ±0.044	0.50 ±0.019	2.002 ±0.041	1.641 ±0.040
	8	7.0 ±4.5E-06	2.38 ±0.026	0.52 ±0.016	2.048 ±0.021	1.705 ±0.035
A. ervi	0	10.2 ±2.0E-06	3.53 ±0.048	0.42 ±0.011	2.543 ±0.038	1.807 ±0.029
	1	10.4 ±1.8E-06	3.58 ±0.048	0.43 ±0.019	2.483 ±0.048	1.763 ±0.050
	2	10.6 ±1.8E-06	3.65 ±0.035	0.41 ±0.014	2.542 ±0.029	1.811 ±0.023
	4	10.4 ±2.1E-06	3.68 ±0.037	0.46 ±0.019	2.468 ±0.026	1.820 ±0.021
	8	10.0 ±5.0E-06	3.68 ±0.036	0.41 ±0.018	2.557 ±0.024	1.872 ±0.021
A. matricariae	0	7.1 ±1.8E-06	2.01 ±0.021	0.39 ±0.010	1.902 ±0.023	1.359 ±0.041
	1	7.1 ±1.8E-06	1.90 ±0.034	0.38 ±0.018	1.828 ±0.028	1.418 ±0.024
	2	7.0 ±1.6E-06	1.89 ±0.032	0.43 ±0.015	1.783 ±0.028	1.540 ±0.032
	4	7.1 ±2.0E-06	1.75 ±0.043	0.38 ±0.016	1.569 ±0.039	1.484 ±0.040
	8	7.5 ±2.2E-06	1.94 ±0.021	0.39 ±0.013	1.792 ±0.024	1.469 ±0.046
A. abdominalis	0	7.8 ±5.1E-06	1.38 ±0.022	0.42 ±0.015	1.146 ±0.053	0.321 ±0.016
	1	7.4 ±3.9E-06	1.38 ±0.021	0.42 ±0.014	0.968 ±0.033	0.330 ±0.016
	2	7.7 ±3.9E-06	1.34 ±0.017	0.44 ±0.014	1.148 ±0.023	0.400 ±0.012
	4	7.2 ±4.0E-06	1.45 ±0.042	0.41 ±0.012	1.228 ±0.031	0.396 ±0.022
	8	7.8 ±3.9E-06	1.33 ±0.016	0.44 ±0.012	0.982 ±0.019	0.387 ±0.020
E. cerasicola	0	8.3 ±3.9E-06	1.74 ±0.019	0.51 ±0.014	1.516 ±0.012	1.342 ±0.020
	1	8.1 ±3.5E-06	1.71 ±0.028	0.52 ±0.018	1.500 ±0.025	1.360 ±0.021
	2	8.8 ±3.3E-06	1.77 ±0.024	0.52 ±0.011	1.498 ±0.021	1.318 ±0.019
	4	8.3 ±3.5E-06	1.76 ±0.018	0.53 ±0.014	1.496 ±0.015	1.310 ±0.021
	8	7.4 ±3.8E-06	1.76 ±0.013	0.54 ±0.013	1.471 ±0.014	1.297 ±0.020
P. volucre	0	7.0 ±2.8E-06	2.01 ±0.02 1	0.39 ±0.010	1.881 ±0.029	1.402 ±0.036
	1	7.1 ±2.0E-06	1.90 ±0.034	0.39 ±0.016	1.821 ±0.028	1.421 ±0.030
	2	7.2 ±1.7E-06	1.89 ±0.032	0.43 ±0.017	1.789 ±0.029	1.537 ±0.032
	4	7.0 ±2.5E-06	1.75 ±0.043	0.39 ±0.019	1.574 ±0.037	1.491 ±0.042
	8	7.6 ±2.4E-06	1.93 ±0.025	0.42 ±0.027	1.797 ±0.036	0.035 ±0.049

Table S8 The percentage of female primary parasitoids that emerged from the aphids that were parasitised during the experiment using washed leaves with hyperparasitised mummies present (*N = 20*)

	Percentage female of emerged primary parasitoids						
	A. colemani	***A. ervi***	***A. matricariae***	***A. abdominalis***	***E. cerasicola***	***P. volucre***	**Hyperparasitoid No.**
0	45.4	53.0	49.3	50.9	44.6	51.9	
1	51.7	49.8	43.7	43.7	48.6	46.5	
2	53.3	45.8	53.3	55.6	52.1	50.0	
4	54.1	53.3	51.2	54.8	51.9	50.9	
8	55.6	45.2	48.9	51.9	52.7	45.2	

Table S9 The measurements of the primary parasitoids that emerged from the aphids that were parasitised during the experiment of washing leaves after adult hyperparasitoid exposure and reapplying the solution, showing the mean weight, body length, head width, hind leg length and antenna length (standard error inclusive) (*N* = 20).

Species	**Hyper-parasitoid number**	**Mean weight (µg ± SE)**	**Mean head width (mm ± SE)**	**Mean body length (mm ± SE)**	**Mean leg length (mm ± SE)**	**Mean antenna length (mm ± SE)**
A. coleman i	0	6.9±3.2 E-06	0.52±0.019	2.58±0.041	2.21±0.045	1.63±0.048
	2	8.0±3.0 E-06	0.53±0.023	2.50±0.026	2.13±0.017	1.84±0.024
	8	6.9±4.5 E-06	0.52±0.016	1.76±0.025	1.50±0.053	1.32±0.016
A. abdominalis	0	7.8±5.1 E-06	0.53±0.015	2.50±0.022	2.13±0.021	1.84±0.043
	2	7.7±3.9 E-06	0.42±0.013	1.39±0.015	1.15±0.014	0.32±0.036
	8	7.8+3.9 E-06	0.44±0.012	1.34±0.016	0.98±0.019	0.39±0.016
E. cerasicola	0	8.3±3.9 E-06	0.52±0.014	1.74±0.02	1.52±0.029	1.34±0.012
	2	8.8±3.3 E-06	0.52±0.011	1.77±0.024	1.50±0.021	1.32±0.2
	8	7.4±3.8 E-06	0.52±0.013	1.77±0.013	1.47±0.023	1.30±0.032
P. volucre	0	7.0±8.2 E-06	0.39±0.01	2.02±0.021	1.88±0.053	1.40±.02
	2	7.2±1.7 E-06	0.44±0.017	1.90±0.032	1.79±0.029	1.54±0.019
	8	7.6±2.1 E-06	0.40±0.015	1.95±0.021	1.80±0.029	1.45±0.035

Table S10 The percentage of female primary parasitoids that emerged from the aphids that were parasitised during the experiment of washing leaves after adult hyperparasitoids were present and reapplying the solution to a clean leaf (*N = 20*).

	Percentage female of emerged primary parasitoids						
	A. colemani	***A. ervi***	***A. matricariae***	***A. abdominalis***	***E. cerasicola***	***P. volucre***	**Hyperparasitoid No.**
0	55.0	53.0	43.8	50.9	45.2	51.9	
1	52.3	49.8	43.7	43.7	48.6	46.5	
2	53.3	53.3	53.3	51.9	50.0	52.2	
4	51.7	52.0	47.9	54.8	51.9	50.9	
8	43.7	45.2	48.9	54.7	52.7	45.2	

Table S11 The measurements of the primary parasitoids that emerged from the aphids that were parasitised during the experiment of washing leaves with hyperparasitised mummies on and reapplying the solution to a clean leaf, showing the mean weight, body length, head width, hind leg length and antenna length (standard error inclusive) (*N* = 20).

Species	Hyperparasitoid number	Mean weight (µg ± SE)	Mean head width (mm ± SE)	Mean body length (mm ± SE)	Mean leg length (mm ± SE)	Mean antenna length (mm ± SE)
A.colemani	0	6.9±E-06	0.52±0.051	2.58±0.045	2.21±0.048	1.63±0.048
	2	8.4±E-06	0.53±0.022	2.50±0021	2.13±0.019	1.84±0.032
	8	7.1±E-06	0.52±0.014	2.38±0.013	2.05±0.021	1.71±0.019
A. abdominalis	0	6.9±E-06	0.42±0.016	1.39±0.032	1.15±0.014	0.32±0.012
	2	7.2±E-06	0.44±0.010	1.35±0.021	1.15±0.029	0.40±0.024
	8	8.4E±06	0.44±0.023	1.34±0.017	0.98±0.048	0.39±0.048
E. cerasicola	0	7.4±E-06	0.52±0.015	1.74±0.016	1.52±0.023	1.34±0.020
	2	8.8±E-06	0.52±0.017	1.77±0.014	1.50±0.022	1.32±0.019
	8	7.7±E-06	0.52±0.011	1.77±0.022	1.47±0.031	1.30±0.036
P. volucre	0	7.2±E-06	0.39±0.015	2.02±0.026	1.88±0.028	1.40±0.020
	2	6.7±E-06	0.44±0.023	1.90±0.025	1.79±0.012	1.54±0.019
	8	7.6±E-06	0.40±0.022	1.95±0.013	1.80±0.053	1.45±0.020

Table S12 The percentage of female primary parasitoids that emerged from the aphids that were parasitised during the experiment of washing leaves with hyperparasitised mummies were present and reapplying the solution to a clean leaf (*N = 20*).

	Percentage female of emerged primary parasitoids					
Hyperparasitoid No.	***A. colemani***	***A. ervi***	***A. matricariae***	***A. abdominalis***	***E. cerasicola***	***P. volucre***
0	43/9	53.0	43.8	51.9	45.2	46.5
1	52.3	49.8	43.7	43.7	48.6	51.8
2	53.4	53.3	53.3	51.9	50.9	52.2
4	43.7	54.8	47.9	52.0	52.7	50.0
8	51.9	45.2	48.9	51.1	54.7	45.2

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