TRITROPHIC INTERACTIONS BETWEEN THE LEAF MINER, *LIRIOMYZA BRYONIAE* (KALTENBACH) (DIPTERA: AGROMYZIDAE) AND THE PARASITOID, *DIGLYPHUS ISAEA* (WALKER) (HYMENOPTERA: EULOPHIDAE).

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ABSTRACT

Liriomyza bryoniae is an economically important pest of vegetable and ornamental crops in European glasshouse agriculture. *Diglyphus isaea* is a parasitoid of *Liriomyza* leaf miners and is commercially available as a biological control agent. Anecdotal reports made to commercial producers of the parasitoid suggest that the efficacy of *D. isaea* varies between crops. This study examines the tritrophic interactions between crop plant, *L. bryoniae* and *D. isaea*.

Host plant was found to influence the abundance of *L. bryoniae* and *D. isaea* with larger populations establishing in the culturing host than in the novel host, tomato. Individual size of *L. bryoniae* also varies with host plant. These patterns are consistent in *L. bryoniae* across three generations of rearing on tomato. Habituation of *L. bryoniae* to tomato does not affect *D. isaea* efficacy nor does the natal plant host of *D. isaea*. Both *L. bryoniae* and *D. isaea* are affected by plant host ontogenetic stage, becoming most numerous on juvenile plants. The *D. isaea* natal insect-plant complex showed no effect on *D. isaea* olfactory preferences. *Diglyphus isaea* demonstrated greater thermal tolerance than its host. These results are discussed in relation to biological control and also in terms of their wider ecological implications.

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1. GENERAL INTRODUCTION

Agromyzid leaf miners are a serious pest of vegetable and ornamental crops worldwide. In Europe one of the more serious leaf mining pests is *Liriomyza bryoniae*, the tomato leaf miner. Since the 1960s, inappropriate use of chemical insecticides has depleted populations of natural enemies, e.g. parasitic Hymenoptera, and has led to the development of resistance in some pest populations (Ho & Ueno, 2007; Minkenberg & van Lenteren, 1986). This, coupled with increased regulation of chemical pesticides (Collier & van Steenwyk, 2004) and increased public concern about the possible effects of chemical pesticides on consumers and the environment (e.g. Carson, 1962), has created a need and a market for effective biological control of insect pests. *Diglyphus isaea* has been used as a biological control agent against *L. bryoniae* since the mid-1980s (Malais & Ravensberg, 2003); however, whilst the control provided by *D. isaea* was initially thought to be excellent, confidence has declined over the past decade. This chapter will review biological control and the current pest status of *L. bryoniae* and its control by *D. isaea* before discussing how and why problems with *D. isaea* may have arisen.

1.1. Biological Control

Over the last century biological control has been defined numerous times; the broadest definition is provided by Bale *et al.*, (2008) who defined biological control as "the use of an organism to reduce the population of another organism". A more utilitarian definition comes from DeBach (1964) in which biological control is defined as "the study and utilisation of

parasites, predators and pathogens for the regulation of host population densities". DeBach also provides an alternative ecological definition in which biological control is "the action of parasites, predators or pathogens in maintaining another organism's population density at a lower average than it would occur in their absence." All these definitions rely on the same basic principle: in nature, most organisms are consumed by other organisms (Bale *et al.*, 2008). Biological control is a corner stone of Integrated Pest Management (IPM), a method of pest control which seeks to "minimise the disturbance of the control effect on the natural component of the agro-ecosystem" (Altieri, Martin, & Lewis, 1983).

Beneficial arthropods have been used in pest control for many centuries, with the earliest authenticated records coming from 4th century China. Hsi Han's *Nan Fang Tshao Mu Chuang* (Records of the Plants and Trees of the Southern Regions), dated 304 AD, refers to the selling of 'reddish-yellow' ants at markets which were used to protect mandarin orange trees from harmful insects. The use of Yellow Citrus Ants *Oecophylla smaragdina* continued in the orchards of Kuangtung until at least the 1960s when they were displaced by chemical pesticides (Hsing-Tsung, 1986). Hsing-Tsung (1986) also refers to a possible earlier record of ants being used for biological control in the Middle East from the 2nd century, although no reference for this is given.

1.1.1. Advantages and Disadvantages

An analysis of the literature suggests that biological control has numerous advantages over chemical pesticides but that these can be countered with a number of scenarios in which chemical pesticides maybe preferable. A significant advantage of biological control is that unlike with chemical pesticides, target species should not be able to develop resistance (Bale

et al., 2008; Blockmans, 1999; van Lenteren, 2000). As biological control has the capacity to be highly specific to a single pest or a small group of closely related pests, it is also compatible with the use of insect pollinators, such as bumble bees, which would be negatively affected by insecticides (Bale *et al.*, 2008; Blockmans, 1999). However, whilst this is of great importance for fruiting crops such as tomato or cucumber, the production of leafy vegetables and many ornamentals does not require pollination. The use of biological control also provides an alternative to pesticides which raise environmental concerns or are subject to regulation (Bale *et al.*, 2008). Further to which, whilst increased regulation has caused the registration of many pesticides to be withdrawn, the number of different biological control agents available is increasing (Warner & Getz, 2008). In the burgeoning market for new methods of pest control the developmental costs of biological control agents is less expensive than for equivalent pesticides (Bale *et al.*, 2008). The application of biological control agents is also less expensive and time consuming than for chemical pesticides, combined with the fact that no safety period is required between application and harvesting, where as a gap of several days is often required for chemical pesticides, which makes biological control an attractive option for growers (Bale *et al.*, 2008; van Lenteren, 2000). Finally, many consumers have become more selective about the produce they buy, and unlike the application of liquid pesticides, biological control agents leave no visible residue on the crops (Gullino & Wardlow, 1999).

Biological control however is not without its critics and nor is it 'problem free'. Generally biological control aims to regulate pest numbers at a reduced level rather than to eradicate the pest entirely, and this may be a concern in two respects. Firstly, with invasive pests, eradication may be preferable (Bale *et al.*, 2008); and secondly those same consumers who

are concerned about pesticide residues on food crops may not tolerate high levels of insect damage to food or ornamental crops. Whilst the development of new biological control agents may be relatively inexpensive their slow acting nature, short shelf lives and the fact that they cannot be patented, limits profitability from biological control agents (Bale *et al.*, 2008; van Lenteren & Woets, 1988). Whilst biological control has the potential to be highly specific to the pests targeted, there is the potential for non-target impacts such as competitive displacement of native natural enemies, interference with native species through intraguild interactions causing a reduction in the distribution or abundance of native species, hybridization with local natural enemies leading to a loss of biodiversity, and vectoring of pathogens. Fortunately, all of the above are rare, having occurred in less than one per cent of biological control introductions, and the use of environmental risk assessment can reduce the likelihood of such impacts in the future (Croy & Myers, 2000; van Lenteren *et al.*, 2006).

1.1.2. Classification

Biological control has commonly been subdivided into three distinct approaches: classical, augmentative and conservation, each of which has a distinct aim, definition and history.

1.1.2.1. Classical control

Classical biological control is the practice of importing and releasing non-indigenous natural enemies, with the aim of establishing permanent populations, for long term suppression of accidentally introduced non-native pests (Liu *et al.*, 2009; Minkenberg, 1990). This method of biological control has been practiced for over 120 years, first starting in the citrus groves of California in 1889.

Following the gold rush of the early 19th century agriculture became the main industry in California with the growing of citrus fruits as a valuable part of the region's economy. However, an accidental introduction of cottony-cushion scale (*Icerya purchasi*) at some point before 1887 devastated the citrus industry. The United Sates Californian entomologist, C.V. Riley, sent Albert Koebele to Australia in search of natural enemy species which preyed on the cottony-cushion scale. In October 1888 Koebele discovered the Vedalia beetle (*Rodolia cardinalis*) feeding on the scales in North Adelaide and dispatched a population of only 129 individuals of *R. cardinalis* to citrus growers in California. This population was maintained over winter and in April 1889 it was released into an orchard and by June of that year 10,555 individuals had been distributed to 228 different orchards (Caltagirone & Doutt, 1989). This early example of classical biological control proved hugely successful. However, classical biological control is also the form which has provided the most serious environmental problems. An example described by Cory and Myers (2000) is that of the *Cactoblastis cactorum* moth, originally from South America, which was imported to Australia in the early 1900s to control invasive cacti. After its success in Australia it was then exported to the Caribbean, from where some individuals reached Florida and attacked the rare native semaphore cactus, *Opuntia spinosissima*.

1.1.2.2. Augmentative control

Augmentative biological control refers to "the release of a large number of insectary-reared natural enemies with the goal of "augmenting" natural enemies populations or "inundating" pest populations with natural enemies" (Collier & van Steenwyk, 2004). Augmentative biological control is divided again by Stinner (1977) into inoculative and inundative. Inoculative releases are those involving "a relatively small number of beneficial arthropods

as colonizing populations, with the purpose of providing relatively long-term pest regulation through in-field reproduction of the released species." Inundative release is the opposite of inoculative, as control agents are released in large numbers "to cause an immediate and direct mortality in the pest population with no expectation of long-term regulation." From these definitions Minkenberg and van Lenteren (1986) re-defined inoculative biological control into two classes: Classical Inoculative biological control, where permanent control is established, and seasonal inoculative control where the release of new agents occurs at the start of each season/crop. The latter of these methods is the one most suited to use in greenhouse vegetables, whilst the former may be used for ornamental greenhouse crops which are cultivated throughout the year.

1.1.2.3. Conservation control

Landis *et al.* (2000) describe conservation control as involving "manipulation of the environment to enhance the survival, fecundity, longevity and behaviour of natural enemies to increase their effectiveness", to which it might be added that these would be indigenous natural enemies. Landis *et al.* (2000) goes on to say however that this cannot simply be achieved by ceasing to use broad spectrum pesticides and that in order for successful pest control to be achieved, habitats which support natural enemies must be spatially and temporally available. There are three major themes in conservation biological control: firstly reducing disturbance through activities such as cover cropping, intercropping and reduced tillage; secondly, supplementing resources through activities such as building beetle banks and planting perennial flowering plants along borders and margins of fields; and thirdly, through reducing predation of natural enemies by secondary enemies e.g. hyperparasitoids

such as *Chrysocharis pentheus* which hyperparasities *Diglyphus isaea* in Japan (Takada and Kamijo 1979 cited in Minkenberg & van Lenteren, 1986).

1.1.3. Other Considerations

Australia became the first country to have any legislation concerning the import of exotic biological control agents when in 1908 the Quarantine Act was introduced and is now the only country to have passed a specific Biological Control Act (Hunt *et al.*, 2008). At the current time Australia, Canada, New Zealand and the U.S.A. all have stringent rules regarding the import of exotic biological control agents. Both Australia and New Zealand, being island states have their own sets of regulations, whilst the approach taken by Canada and the U.S.A. is coordinated by the North American Plant Protection Organisation (NAPPO) (Hunt *et al.*, 2008). The regulation of biological control has not yet been harmonized in Europe, although the need for a Europe-wide approach has become clear since the introduction of the Multicoloured (Harlequin) Asian Lady Beetle *Harmonia axyridis*. The introduction of *H. axyridis* was prohibited by regulatory authorities in Switzerland; however, after subsequent release in neighbouring countries with no regulation (Belgium and France) and with no way to prevent it from crossing national borders, it has now invaded Switzerland (Bigler *et al.*, 2005). As signatories to the Convention on Biological Diversity (http://www.cbd.int/) most European countries are required to have controls on the introduction of alien species (C.B.D., 1992); however, six countries (Belgium, France, Greece, Italy, Poland and Portugal) have no regulation. With no uniform approach across Europe, organisations wishing to register a new biological control agent may have to apply multiple times in different formats to multiple regulatory bodies, thus escalating the cost of biological control in Europe (Bigler *et al.*, 2005).

1.2. Study System

1.2.1. *Liriomyza bryoniae* (Kaltenbach)

Liriomyza bryoniae (Kaltenbach) (Diptera: Agromyzidae) synonymous with *L. solani* (Hering), *L. citrulla* (Rohdendorf) and *Agromyza bryoniae* (Kaltenbach), is also colloquially known in English as the tomato leaf miner (EPPO, 2012). A native European species (Rice Mahr *et al.*, 2001), established in Great Britain (but not Northern Ireland, (DEFRA, 2007)), mainland Europe, North Africa, the Middle East, continental Asia, Taiwan and Japan (EPPO, 2012) as a pest of vegetables, especially tomatoes (Minkenberg & Helderman, 1990). *L. bryoniae* is polyphagous and as such can feed indiscriminately on plants from a number of different orders, including many vegetable and ornamental species (Minkenberg & van Lenteren, 1986). Few authors have discussed the wild plants that *L. bryoniae* utilises as hosts, although, Gil-Ortiz *et al.* (2008) report the occurrence of *L. bryoniae* in London rocket, *Sysimbrium irio* L. (alternative spelling *Sisymbrium irio*), a wild European winter annual belonging to the *Brassicaceae* family (Ray *et al.*, 2005).

The life history of *L. bryoniae* is typical of the genus, as outlined in Parrella (1987). A fertilized adult female will deposit eggs individually through holes made in a leaf, formed by the thrusting of the female's ovipositor through either surface, adaxail or abaxail, of a leaf and into the mesophyll. In this way females form two types of leaf puncture: a fan shaped puncture used only for feeding, and a tubular puncture used for both feeding and oviposition (Parrella, 1987). Each oviposition leaf puncture contains just one opaque ellipsoidal egg (Minkenberg & van Lenteren, 1986). Three larval instars develop within the leaf and by continued feeding mine their way through the leaf until the final larval instar cuts

a semi-circular exit hole through the leaf surface (Parrella, 1987). The prepupal larvae leaves its mine and pupates in the soil at the base of the plant at a depth of approximately 5cm (Minkenberg & van Lenteren, 1986; Parrella, 1987). Pupal colour ranges from light yellow to dark-brown (Malais & Ravensberg, 2003). Adults emerge from pupae by inflating the ptilinum (Parrella, 1987); once fully sclerotized, adult *L. bryoniae* have a wing length ranging from 1.7 to 2.1mm, and are predominantly black and yellow with a black mesonotum and yellow thoracic shield (Malais & Ravensberg, 2003; Spencer, 1972). The genders of *L. bryoniae* are easily identifiable as females possess a pointed black tip to their abdomen which the males lack (Minkenberg & van Lenteren, 1986). Total development time from egg to adult emergence in tomato takes 26.5 days at 20°C (Minkenberg & Helderman, 1990). Minkenberg and Helderman (1990) showed that the optimum temperature for *L. bryoniae* development is 25°C whilst the minimum temperature is 8°C. Adult longevity in males is typically less than three days, whilst females survive for approximately nine days at 20°C (Minkenberg & Helderman, 1990; Minkenberg & van Lenteren, 1986).

Damage to plants by *L. bryoniae* is caused in two ways: firstly, through feeding and oviposition holes made in the surface of leaves by female leaf miners, and secondly, by the mines hollowed out in the mesophyll of the leaves by the developing larvae (Minkenberg & van Lenteren, 1986; Parrella *et al.*, 1985). This damage causes decreased photosynthesis, increased desiccation, premature leaf fall and under certain conditions, the sun burning of fruits (Minkenberg & van Lenteren, 1986). Damage can also be caused indirectly through fungal and bacterial infections entering the plant through feeding and ovipostion holes and through the transportation of plant viruses (Minkenberg & van Lenteren, 1986). Some plants are able to cope surprisingly well despite the damage caused by *Liriomyza* leaf miners. For

instance, Johnson *et al.* (1983) suggest that whilst mining by *L. sativae* causes a 62% reduction in photosynthetic rates in mined tomato leaflets compared with unmined leaflets, the photosynthetic potential of a tomato plant is sufficiently greater than its demand for energy that leaf miner damage is unlikely to cause a decrease in fruit production. Indeed, tomato seems to be able to withstand 25% defoliation before a significant loss in yield will occur (Stacey, 1983). Plants which are able to cope with low levels of infestation are ideal candidates for biological control.

Ferguson (2004) demonstrated that *L. trifolii* caught from populations living on ornamentals under greenhouse production in the U.S.A. had developed moderate to high levels of resistance to the insecticides cyromazine, abamectin and spinosad. However, it has been known since the 1950s, when Hills and Taylor (1951) showed that repeated applications of DDT to *Liriomyza* infested cantaloupe were ineffective, that this genera can readily evolve pesticide resistance. It is therefore essential to develop alternative methods for controlling *Liriomyza* leaf miners and biological control could be a viable alternative. However, very few predators have been recorded for this genus; in their review of Integrated Pest Management (IPM) of *Liriomyza* Murphy and LaSalle (1999) reported that predatory empidid, mucid and dolichopodid flies and one species of nematode had been found to prey on *Liriomyza spp.* However, over 140 species of parasitoids are known to use *Liriomyza* as hosts (Liu *et al.*, 2009) and of these, several species may be suitable for use as biological control agents including *Diglyphus isaea* and *Dacnusa sibirica*.

1.2.2. *Diglyphus isaea* (Walker)

Diglyphus isaea (Walker) (Hymenoptera: Eulophidae) is a species of primary larval ectoparasitoid of leaf minning Diptera. *Diglyphus isaea* has at least 16 synonyms (Noyes, 2012) and is marketed with the commercial pseudonym Miglyphus (www.koppert.com). Originally described in Britain (Walker, 1838) and found throughout Europe, North Africa and Japan, this holartic species has now become cosmopolitan after being introduced to the U.S.A., Canada and New Zealand (Minkenberg, 1989). *Diglyphus isaea* is a synovigenic species; the females sequentially produce eggs throughout their adult lives, and, so as to meet the energetic costs of egg maturation, host feeding is required (Giron *et al.*, 2004). Host feeding is a major factor contributing to the mortality of *D. isaea* hosts (Minkenberg, 1989). Once fed, *D. isaea* females deposit a single cylindrical egg next to a paralyzed late instar larval *Liriomyza* host; when they have emerged from eggs, larval *D. isaea* proceed to consume their host and pass through three distinct larval stages before moving off down the mine to pupate (Ibrahim & Madge, 1979; Minkenberg & van Lenteren, 1986). *Diglyphus isaea* remains within the leaf until adult eclosion, when the adult cuts through from the mine to the leaf surface to escape (Minkenberg & van Lenteren, 1986). The genders of *D. isaea* are easily identifiable as the hind tibia of the male is marked with a pair of small dark bands whereas the female has only a single large dark band (Askew, 1968). Development time (egg to adult emergence) at 20°C typically takes 16 – 17 days; this is approximately 10 days less than *L. bryoniae* at the same temperature and on the same host plant (Minkenberg & Helderman, 1990; Minkenberg, 1989). Adult longevity at 20°C is approximately 32 days (Minkenberg, 1989).

Since 1984 *D. isaea* has been a widely used biological control agent employed against leafmining pests including *L. bryoniae* and also *L. trifolii* and *L. huidobrensis*, which are also pest species in glasshouse agriculture (Malais & Ravensberg, 2003). *Diglyphus isaea* is often sold in combination with *Dacnusa sibirica* and various release rates are recommended depending on the prevailing conditions and the level of leaf miner infestation (www.koppert.com). Mass rearing of *D. isaea* for biological control purposes takes place on the leaf miner host *L. trifolii* feeding in French bean (*Phaseolus vulgaris*). The leaf miner adults are allowed to oviposit onto the bean plants for 48 hours before being removed. Once the leaf miner larvae have reach the third instar, the *D. isaea* adults are released into the cages and allowed to parasities the leaf miners, at 25ºC and RH70%; 150 parasitoids are introduced to every 250 leaf miner adults, from which 500 – 700 *D. isaea* can be harvested weekly (Onillon, 1999).

1.2.3. Tritrophic System

The three-way, tritrophic, interactions of plant, herbivore and natural enemy are central to our understanding of this system or indeed any terrestrial community (Price *et al.*, 1980). However, due to the polyphagous nature of *L. bryoniae*, several different plant hosts have been utilised during the course of this study; each plant host will therefore be introduced and discussed, as this information becomes relevant in the experimental sections of this work.

1.3. Current Control System

The current status of worldwide *Liriomyza* control was discussed in a project mapping meeting with the industrial CASE partner (Koppert Biological Systems) in December 2008. The information below is based in part on reports, observations and anecdotal accounts (from growers and representatives of Koppert), and therefore represents the most up to date view of the efficacy of current biological control programmes for *Liriomyza spp.*

1.3.1. Northern Europe

During winter, leaf miners are found in glasshouses in low numbers and *Dacnusa sibirica* (a pro-ovigenic, endoparasitoid) provides an adequate level of control. When temperatures increase in early spring, leaf miner populations increase rapidly and *D. sibirica* is unreliable at maintaining control, and *D. isaea* is not sufficiently cold-adapted at this time to supplement the control provided by *D. sibirinca.* A population of *D. isaea* only starts building up when temperatures rise to about 20 °C, usually in April.

Until the end of the 1990s leaf miner control in tomatoes was considered to be good (Benuzzi & Raboni, 1992 cited in Nedstam & Johansson-Kron, 1999), but control has subsequently declined. However, reports also suggest that in other crops such as ornamental gerbera, leaf miners are adequately controlled by *D. isaea* during winter. Gerbera is a perennial ornamental and is continuously cultivated throughout the year for the cut flower market. It would appear that *D. isaea* has become self-sustaining in gerbera glasshouses; however, these populations may be a mix of the descendants of earlier releases and wild *D. isaea*.

1.3.2. Southern Europe and North Africa

In Spain, the control situation is better than in the north, but is still insufficient in winter. Anecdotal reports indicate that on the Canary Islands *D. isaea* usually provides adequate control during winter. In Turkey and Tunisia, leaf miners are not a problem as they are kept in check by wild *Digylphus spp.*. It is possible that wild *Diglyphus spp.* are also responsible for control in the Canary Islands and those areas of Spain where control appears to be effective.

1.3.3. North America

In Canada and the northern U.S.A. leaf miners are not a major problem in tomato, whilst in the southern U.S.A. and in Mexico other *Diglyphus spp.* occur spontaneously year round and there is no need for augmentative release. However, where augmentative release of *D. isaea* has been used, it was recently demonstrated that when the wasps were recaptured and identified to species, no *D. isaea* were found but that *D. beginii* was the most abundant species of parasitoid in the glasshouses (pers. comm. J. Klapwijk).

1.4. Possible Explanations

There are four broad areas within the *D. isaea*-*L. bryoniae*-biocontrol arena that are possible explanations for the decline in leaf miner control, as outlined in Figure 1-1. These include: 1) the conditions *D. isaea* is exposed to in the modern winter glasshouse environment, both the climatic conditions and the chemical conditions such as raised atmospheric CO_2 concentration. 2) The species and cultivar of crop plants with their associated defenses. 3) The strain, race or species of leaf miner. 4) The system the parasitoids are cultured under and the possibility of habituation to the host used in production.

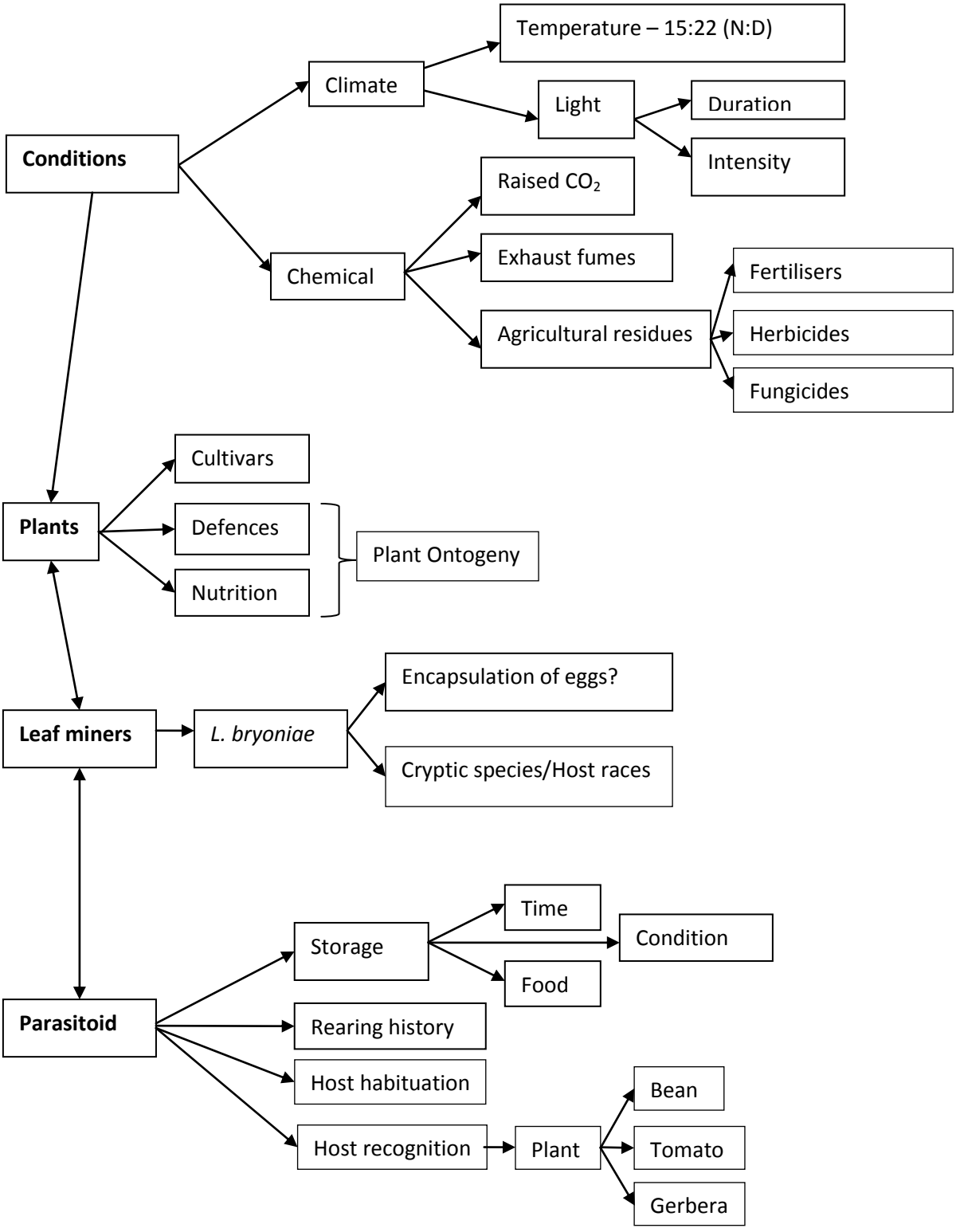

Figure 1-1: Summary of the interacting factors in the control of *Liriomyza* leaf miners with *D. isaea*.

1.4.1. Environmental Conditions

The chemical conditions in modern glasshouse environments can differ from the natural environment in several ways. Conditions can be altered through the addition of substances intended to increase crop yield, such as fertilisers or gaseous CO_2, or through the addition of substances intended to prevent/reduce damage to the crop caused by insects, fungi or other pests. Very little is known about the effects that fertilisers, herbicides or fungicides might have on parasitoid wasps (Liu *et al.*, 2009), and virtually nothing about what effects they may have in combination. However, it has been known for some time that insecticides can have a negative effect on parasitoid species. This was first shown by Hills and Taylor (1951) who commented on the negative impact of DDT on natural enemies and how this led to an outbreak of leaf miners in an experimental crop of cantaloupe melons. Some modern broad spectrum insecticides such as cyromazine and abamectin are also known to adversely affect parasitoid species such as *D. isaea* (Kaspi & Parrella, 2005; Weintraub, 2001). For instance, the insecticide/miticide abamectin has been shown to have a negative effect on adult *D. isaea* and to be lethal to *D. isaea* larvae, both when applied directly and when consumed through contaminated *Liriomyza* hosts (Weintraub, 2001). The use of pesticides in glasshouse agriculture is however often still necessary to control a broad range of pests not targeted by currently available commercially biological control agents.

The stability of climatic conditions inside glasshouses allows those organisms adapted to the prevailing conditions, and not requiring seasonality, to complete their life cycle. However, this allows many pest species to persist in locations at greater latitudes than field populations of the same species. Similarly, natural enemies can potentially over winter or be introduced from milder climates than those available outside the glasshouse (Kang *et al.*,

2009). Relatively small changes in temperature, within the scope of those that may be experienced in a glasshouse, can however impact important life history variables such as developmental rate, longevity and fecundity (number of eggs laid); for both *L. bryoniae* and *D. isaea*, these variables have been previously studied (Minkenberg & Helderman, 1990; Minkenberg, 1989). Other aspects of the thermal biology of these species however are yet to be quantified including non-lethal measures of the critical thermal limits (CTmin and CTmax), the low and high temperatures beyond which an insect loses the capacity for coordinated movement (Sinclair *et al.*, 2006), and coma inducing temperatures, defined by the high or low temperature at which the last twitch of an appendage occurs (Hughes *et al.*, 2010). Ideally a predator or parasitoid should have a broader thermal tolerance range than its prey/host; a biological control agent capable of maintaining coordinated movement at temperatures beyond which its target has been immobilised would be more likely to provide effective control both in the spring when temperatures may still be low and young plants are vulnerable and during high summer temperatures as crop mature.

1.4.2. The Pest

There are several potential problems with our understanding of the pest-parasitoid interaction. The most obvious problem could be that the *Liriomyza* species against which *D. isaea* is employed is not *L. bryoniae*. A more subtle issue could be that *L. bryoniae* is not a single species but a complex of cryptic species and that *D. isaea* is not compatible with some or all of these. Alternatively, it is possible that *L. bryoniae* is able to defend itself in some way against the *D. isaea* larvae.

There are over 300 species of *Liriomyza* leaf miner, of which 23 are considered to be economically important (Parrella, 1987), whilst of these, only six are polyphagaus: *L. bryoniae, L. sativae* (Branchard), *L. trifolii* (Burgess), *L. huidobrensis* (Branchard) *L. strigata* (Meigen) and *L. longei* (Frick) (Liu *et al.*, 2009). Most of these *Liriomyza* species are originally from the New World; however, anthropomorphic activities have caused them to be spread to most of the world's temperate and tropical regions (Liu *et al.*, 2009). Both *L. trifolii* and *L. huidobrensis* have become significant pests in Europe; unfortunately these two species are very difficult to differentiate between or from *L. bryoniae* by sight (Malais & Ravensberg, 2003; Weintraub & Horowitz, 1995). The adults of all three species are black and yellow in colour and share the same size range of 1.5 – 2.5 mm in length. *Liriomyza trifolii* may be identifiable by its yellow larvae, whereas both of the other two species have transparent/milky white larvae; however, all three species produce a variety of different coloured pupae (Malais & Ravensberg, 2003). *Diglyphus isaea* is known to be capable of parasitizing both *L. trifolii* and *L. huidobrensis* (Bazzocchi *et al.*, 2003; Weintraub & Horowitz, 1995) and indeed it is marketed by Koppert Biological Systems as a biological control agent against these leaf miner species.

Liriomyza bryoniae, *L. trifolii* and *L. huidobrensis* although sometimes indistinguishable to the naked eye, can be separated morphologically by the structure of the male genitalia (Malais & Ravensberg, 2003). It is possible however that there are separate but morphologically indistinguishable forms of *L. bryoniae*, termed cryptic, sibling or isomorphic species (Dujardin, 2008). In the absence of any known quantitative trait which can be used to distinguish between such species, genetic techniques are the tool of choice; however, there may also be behavioural or ecological differences between cryptic species. A combination of

behavioural observation and genetic analysis has been used to demonstrate that both *L. trifolii* and *L. huidobrensis* contain cryptic species (Scheffer & Lewis, 2006; Scheffer & Lewis, 2001). It is possible that any genetic, behavioural or ecological differences between cryptic species could have implications for any management strategy including biological control. A possible step on the route to speciation is the formation of biotypes or host races; these are populations which become partially isolated from other populations of the same species due to high levels of host fidelity (Diehl & Bush, 1984). Although *L. bryoniae* has a reputation for being highly polyphagous at the species level it is possible that host races may have formed, especially in glasshouses used for the same crop year on year.

Finally, it is possible that if cryptic species do exist within *L. bryoniae*, some or all of these may have mechanisms by which their larvae can defend themselves against *D. isaea*. If such a mechanism exists, it is likely that it involves the encapsulation of the parasitoid egg or larva. Encapsulation is a process by which the immune system of the host responds to any invading material identified as non-self by surrounding that material with an aggregation of haemocytes which proceed to dispose of the non-self-material by phagocytosis (Gullan & Cranston, 1994). It has been observed that *L. trifolii* is capable of encapsulating *D. sibirica* eggs (Parrella, 1987); however *D. sibirica* is an endoparasite and therefore deposits its eggs into living larvae, whereas *D. isaea* is an ectoparasite and oviposits next to a paralysed leaf miner larva so that the parasitoid larva commences its attack from the outside of the host (Malais & Ravensberg, 2003), making encapsulation unlikely.

1.4.3. The Parasitoid

There are some areas in our understanding of the biology of *D. isaea* relevant to its use as a biological control agent where less information is available e.g. the possibility that, like some species of *Liriomyza*, *D. isaea* is a complex of cryptic species, the effects that long term rearing on bean (*P. vulgaris*) might have had on a biocontrol agent commonly used against pests in tomatoes, the possible effects of storage and transport, and finally, reproductive problems such as a skewed sex ratio or the effects of *Wolbachia*, if present, such as cytoplasmic incompatability.

Recent findings suggest that *D. isaea* is a complex of cryptic species, at least within China. Molecular analysis of the nuclear genetic code of *D. isaea* has revealed the presence of four separate geographically isolated clades (Sha *et al.*, 2007). There are no known morphological differences between the clades but they are sufficiently divergent for them to be considered separate cryptic species. To date, *D. isaea* has not been similarly analysed in Europe but given that Sha *et al.*, (2007) suggest that geographic isolation of the clades in China is likely to be associated with *D. isaea*'s poor flying abilities, it would seem likely that geographic barriers to gene flow would also occur in Europe. It is also possible that divergence could have occurred between those *D. isaea* taken into culture for mass rearing and wild *D. isaea*.

The process of the mass rearing of natural enemies (previously described with reference to *D. isaea*) is a constant conflict between producing the highest possible quality biological control agent and keeping production costs as low as possible. Problems associated with the mass rearing of parasitoids include: genetic deterioration, behavioural changes as a result of rearing on alternative host, and in alternative conditions to those in which it will operate

(Van Lenteren & Tommasini, 1999). The more artificial the nature of the situation under which the parasitoid is reared, the more severe these problems are likely to be.

Genetic deterioration of mass reared parasitoids can lead to changes in parasitoid phenotypes including loss of ability to overcome unexpected stresses, decreased intraspecific competitive abilities, decreased genetic variability due to uniformity of rearing conditions, changes in density-dependent dispersal and searching behaviours (Van Lenteren & Tommasini, 1999). Introducing wild individuals into the culture may seem like an easy way of maintaining genetic diversity; however, as the wild types will be exposed to the same selective pressures as the rest of the culture, any benefit from the wild types will be quickly lost as their descendents become adapted. Further to which the introduction of parasitoids from the wild increases the risk of contamination of the culture or the introduction of pathogens (Van Lenteren & Tommasini, 1999).

Behavioural problems associated with mass reared parasitoids can also occur due to the effect of 'pre-imagnial olfactory conditioning'. This term, coined by Thorpe and Jones (1937), refers to the effect of the environment in which an immature stage is raised on its behaviour in the adult form. Thorpe and Jones (1937) demonstrated how a parasitoid reared on a novel host species could learn that this new species was a potential host for its own young. More recently Turlings *et al.* (1993) have shown that emerging adults of the leaf miner parasitoid *Opius dissitus* reared on hosts in lima bean prefered leaf miner larvae in lima bean as oviposition sights over leaf miner larvae in egg plant. The idea of 'pre-imaginal conditioning' therefore implies that whilst parasitoids are developing as larvae, they learn chemical cues from their environment, host insect and plant, which as adults they then search for as

preferred oviposition sites (Prokopy & Lewis, 1993; Thompson, 1999; Turlings *et al.*, 1993). Further to which Prokopy and Lewis (1993) suggest that the artificial conditions in a mass production facility can lead to distorted behavioural profiles in adult biological control agents and that in order to prevent this, it may be necessary to introduce key semiochemicals into the rearing environment.

After rearing under commercial conditions it may be desirable or necessary to store the control agents for some time; however, this is generally thought to lead to reduced fitness, especially where they are stored in the adult stage (Van Lenteren & Tommasini, 1999). Despite this, it has been demonstrated that *D. isaea* can be stored at low temperature for two months without an increase in mortality or decrease in fecundity (Burgio and Nicoli 1994 cited in van Lenteren & Tommasini, 1999). However, no reference is made to any effect this has on flying capabilities or searching behaviours, both of which could be negatively affected in sustained, confined conditions and in the absence of host cues.

Female Hymenoptera, including *D. isaea*, are able to control the gender of their offspring through 'haplo-diploid determination of sex'. In the case of parasitoid wasps, males are derived from unfertilised haploid eggs whilst the fertilisation of eggs give rise to diploid females (Chow & Heinz, 2005; Flanders, 1956). This is possible because female parasitoids are able to retain the sperm received during mating in a spermatheca and are able to control its release, thus 'choosing' the gender of each of their offspring (Flanders, 1956). In this way female parasitoids are able to determine the ratio of males to females in the next generation. In wild populations, the ability of parasitoids to determine the gender of offspring in variable environmental conditions allows a female to maximise her returns in

terms of fitness (Chow & Heinz, 2005). For biological control purposes however, the over production of male parasitoids (more than are required to sustain a population) is undesirable, as only females can directly contribute to the mortality of pests through host feeding and oviposition (Chow & Heinz, 2005; Ode & Heinz, 2002). It is known that commercial cultures of *D. isaea*, at least in North America, have male biased sex ratio, with 60 – 70% of individuals being male (Heimpel & Lundgren, 2000). Charnov *et al.*, (1981) showed that the parasitoid *Lariophagus distinguendus* produces mostly males in small hosts and mostly females in large hosts, and that large and small are relative rather than absolute measures made by the parent wasp. This has now been demonstrated by Ode and Heinz (2002) to also be the case in *D. isaea*. Ode and Heinz (2002) further examined how female parasitoids make relative judgements about host size and found that the choices made are affected most strongly by the size of hosts recently encountered. This has led to a number of suggestions of ways in which the sex ratio of *D. isaea* under mass production might be manipulated to ensure a more female biased sex ratio - generally through manipulation of host populations to control the range of sizes of hosts encountered by *D. isaea* females (Chow & Heinz, 2005; Ode & Heinz, 2002).

1.4.4. Tritrophic Interactions

There are two ways in which plants can interact with the third trophic level, indirectly through herbivores and directly by influencing the parasitoid's host locating behaviours.

1.4.4.1. Indirect interactions

Minkenberg and van Lenteren (1986) describe *D. isaea* as being associated with *Liriomyza* hosts in herbaceous plants but scarce in trees. *Diglyphus isaea* has certainly been used as a

biological control agent in a wide range of crops, whilst in the wild, populations of *D. isaea* have been found to use at least 14 species of weedy plant in northern Italy (Burgio *et al.*, 2007). It is likely that at least some of the plants represent the native wild host plants of *D. isaea* and its insect hosts. Despite the wide range of *Liriomyza* infested plants that *D. isaea* can be reared from it is likely that some plant-insect host combinations represent a better utility to developing *D. isaea* larvae than others. Salvo & Valladares (2002) have shown that three Argentine parasitoid species, *Phaedrotoma scabriventris* (Nixon), *Halticoptera heliponi* (De Santis) and *Chryocharis flacilla* (Walker), developing on the same insect host species, produced different sized adults in the next generation, depending on the plant host species. This was primarily due to variation in the size of insect hosts between the plant species, as ultimately, a smaller insect host represents a smaller resource to a developing parasitoid, so it can only achieve a smaller adult size relative to a parasitoid developing on a larger host. However, they also found evidence for a host plant specific effect on parasitoid size; despite the host insect achieving an equal size in both common beet (*Beta vulgaris cycla*) and broad bean (*Vicia faba*), the parasitoids developing on beet were consistently smaller than those developing in broad bean. Salvo & Valladares (2002) hypothesized that this is due to semiochemical, chemical or physical properties of the plant, e.g. toxins or allelochemicals acting either directly on the developing parasitoid or indirectly through its host. It is likely that *D. isaea*, which uses a wide range of *Liriomyza* infested plant species, would also experience plant-specific effects in some of its host plants.

1.4.4.2. Direct interactions

There are numerous ways in which a parasitoid could locate its prey from visual, acoustic, gustatory, chemical and touch signals (Kang *et al.*, 2009). Whilst some if not all of these

sensory modes are used by parasitoids, the general consensus in the literature is that long distance detection of hosts occurs though chemical stimuli. Plant volatiles, especially herbivore-induced plant volatiles (HIPVs) are believed to be the dominant signal used by parasitoids in host location and can in some ways be regarded as communication between the first and third tropic levels (Kessler & Baldwin, 2001; Takabayashi & Dicke, 1996).

HIPVs are produced by plants specifically in response to herbivore damage; emission of the same volatile cocktail cannot be evoked from a plant simply through mechanical damage. In an example specifically relevant to the current study, Finidori-Logli *et al.* (1996) showed from gas chromatography that *Phaseolus vulgaris* plants produced a different volatile blend when infested with second instar *L. trifolii* larvae to that produced when artificially damaged. Whilst in a classical example, Turlings *et al*. (1995) discussed how the application of caterpillar regurgitate to mechanical wounds on a plant can produce an identical volatile response to that of caterpillars feeding on them.

The cocktail of volatiles released by plants is not simply specific to the type of damage, (herbivore vs. mechanical); different blends of volatiles are released depending on the species of herbivore attacking the plant. Wei *et al.*, (2006) showed that although *P. vulgaris* plants attacked by *L. huidobrensis* and *L. sativae* emitted the same qualitative mix of volatiles, they emitted them in different volumes depending on which *Liriomyza* species was responsible for the damage. Further to which, parasitoids are able to distinguish between these different volatile blends, as demonstrated by Turlings *et al*., (1993), who showed that *Cotesia marginiventris* is able to express a preference between two species of armyworm, *Spodoptera frugiperda* and *S. exigua*, feeding on the same species of corn.

Although parasitoid volatile detection can be sensitive enough to detect which species of herbivore is attacking a plant, they can also identify volatile blends from other species of plant being attacked by the same herbivore. Turlings *et al*., (1993) have shown that the parasitoid *C. marginiventris* is capable of both responding to and distinguishing between the different volatile blends emitted by corn, cowpea, soybean and cotton, when each plant is fed upon by the same species of armyworm. So, parasitoids are able to identify a number of different volatile blends from different plants and identify whether the presence of suitable host larvae is implied by these different volatile cocktails. It has been demonstrated by a number of authors that *D. isaea* has at least some of these abilities (Finidori-Logli *et al.*, 1996; Zhao & Kang, 2002)

1.4.4.3. Plant ontogeny

There are four distinct phases in plant development: seedling, vegetative juvenile, mature and senescent. The level and type of defences and the nutritional value of the plant change with ontogenetic phase; however, across the plant kingdom there is not uniformity in the direction or onset of these changes (Barton & Koricheva, 2010). These changes are also accompanied by changes in plant size, architecture and root: shoot ratio (Boege & Marquis, 2006) causing herbivores to reach a compromise on plant quality verses quantity. In this way herbivore populations are regulated according to the ontogenetic stage of their host plant. Plants are also able to signal the presence of herbivores to natural enemies, but the production of HIPVs also varies according to the ontogenetic stage of the plant under attack (Rostás & Eggert, 2007). In annual crops the ontogenetic stage of plants will change with the season; it has been reported that the efficacy of *D. isaea* as a control agent of *L. bryoniae*

also varies seasonally, but the importance of plant ontogeny to this seasonal variation has not been previously studied.

1.5. Aims and objectives

Reports that the efficacy of *D. isaea* as a biological control agent of *L. bryoniae* is in decline have been largely anecdotal so the initial aim of this study was to quantify any difference in the ability of *D. isaea* to utilise *L. bryoniae* as a host in a variety of different crops. Once differences in *D. isaea* efficacy were quantified, the subsequent aim of this work was to determine why these differences may have arisen. From the review of the literature presented in this chapter, the likely causes of this variation include:

- Habituation to the culturing insect-host-plant-host complex affecting the ability of *D. isaea* to utilise *L. bryoniae* as a host in target crops.
- Effects of the natal host on the searching behaviour through preference induction.
- Changes in plant ontogeny affecting both the use of the plants by herbivores and the attractiveness of the plants-herbivore complex to *D. isaea*.
- Aspects of *D. isaea* thermal ecology, particularly the lower and upper critical temperatures which constrain normal behaviour.

In studying these four potential causes of variation it was hoped that a clearer pattern of the relationship between *D. isaea* and *L. bryoniae* would emerge, especially with regard to the effect of the plant host and the environment on their interrelationship.

2. INSECT CULTURE

The primary aim of this project is to understand why the biological control of *Liriomyza* leaf miners with *Diglyphus isaea* has become variable between crops. To this end, populations of *D. isaea* and the leaf miner *Liriomyza bryoniae* were maintained in culture in the School of Biosciences at the University of Birmingham, from October 2008 until June 2012.

2.1. Plants

The culture system is tritrophic in nature, consisting of a plant, herbivore and parasitoid. A number of plant species are suitable for use as a host for *L. bryoniae* as this leaf miner is highly polyphagous (Minkenberg & van Lenteren, 1986). However, two host plants were used for most ptojects: broad bean (*Vicia faba*) for the culture of *L. bryoniae* and French beans (*Phaseolus vulgaris*) infested with *L. bryoniae* to culture *D. isaea*. These plant species were chosen in order to replicate the conditions under which *D. isaea* is mass reared by commercial producers of *D. Isaea* for biological control.

2.2. Conditions

The cultures were maintained in a controlled environment room at the University of Birmingham in 60x60x60 cm BugDorm-2120 insect rearing tents (MegaView Science Co., Ltd., Taiwan), under artificial light with a photoperiod of 16:8 (Light:Dark, lights on at 08:00am) and at a constant temperature of 23°C.

2.3. *Liriomyza bryoniae*

The initial culture of *L. bryoniae* was received from Koppert Biological Systems in October 2008, in three shipments each containing approximately 200 pupae, received at one week intervals, allowing three separate cultures to be established so that every life stage of *L. bryoniae* would be available at all times.

2.3.1. Host plant

Liriomyza bryoniae were cultured on broad bean (*Vicia faba* var. Sutton Dwarf) grown at the University of Birmingham, under glasshouse conditions, to maintain a stock population of the leaf miner.

2.3.2. Culture Method

Thirty six broad bean plants were placed into an insect cage into which *L. bryoniae* adults were released and allowed to feed, mate and oviposit freely. *Liriomyza bryoniae* larvae developed within the plant leaves and pupated after approximately 10 days. Under natural conditions, the larvae would have left their mines and pupated in the soil (Malais & Ravensberg, 2003). However, so that the pupae could easily be collected, the plants were laid on their sides, so that the leaf miner larvae fell onto the plastic base of the cage from where they were collected using a soft paint brush. Pupae were stored in Petri dishes on dampened discs of tissue paper in the controlled environment room until the new generation of adults emerged.

2.3.3. The Colony

The generation time from egg to adult of *L. bryoniae* at 23°C was approximately 21 days. So as to have each stage of the life cycle available 'on demand' for experiments, three populations were maintained, each consisting of approximately 200 individuals.

2.4. *Diglyphus isaea*

The initial culture of *D. isaea* was received from Koppert Biological Systems in January 2009; three shipments were received at 2 week intervals, each containing approximately 200 adult *D. isaea*.

2.4.1. Hosts Plants and Insects

D. isaea was maintained on *L. Bryoniae*-infested French bean plants (*Phaseolus vulgaris* var. Tendergreen). French beans were used instead of broad beans because they retain their leaves for longer after infestation with *L. bryoniae*. The French beans were grown at the University of Birmingham under glasshouse conditions.

2.4.2. Culture Method

Thirty six French bean plants were infested with *L. bryoniae* larvae as described above. Once the *L. bryoniae* had attained their 2nd instar, adult *D. isaea* were released into the insect cage and allowed to host feed and parasitize the larval leaf miners. The new generation of *D. isaea* emerged approximately 16 days after oviposition. The newly emerged *D. isaea* displayed a positively phototactic response and attracted by the lights above the Bugdorm, would congregate at the top of the cage from where they were collected using an aspirator.

2.4.3. Storage

Diglyphus isaea can be stored at low temperature for months without an increase in mortality or a decrease in fecundity (Burgio and Nicoli 1994 cited in van Lenteren and Tommasini 1999). For this reason, when necessary, the adult parasitoids were stored at 10°C until suitable plants and *L. bryoniae* larvae were available. Stored parasitoids were used for culturing purposes only.

2.4.4. The Colony

The parasitoid culture was maintained with approximately 100-150 adults in a cage.

3. INTERACTIONS BETWEEN *LIRIOMYZA BRYONIAE AND DIGLYPHUS ISAEA* IN DIFFERENT HOST PLANTS.

3.1. Abstract

Recent anecdotal reports have suggested that the level of *Liriomyza bryoniae* (tomato leaf miner), suppression by the commercially available biological control agent *Diglyphus isaea* varied according to the host plant on which the larval leaf miners were feeding. In order to quantify this variation, a series of experiments utilising three different host plants (tomato, gerbera and French bean), have been conducted. The laboratory strain of *L. bryoniae* infested only tomato and French bean but not gerbera. Survival of *L. bryoniae* from larvae to adult was consistently greater in French bean than in tomato and both *L. bryoniae* pupae and adults were larger when reared from French bean. The number of *D. isaea* in the offspring generation corresponded to the number of *L. bryoniae* larvae. *D. isaea* sex ratios were highly variable, though no impact of plant was seen on the size of *D. isaea* individuals. Possible explanations for the differences seen in *L. bryoniae* and *D. isaea* according to the host plant on which they were reared are discussed.

3.2. Introduction

A biological control system, like a natural plant based ecosystem, contains at least three elements: the plant, the pest and a predator, or in this case, a parasitoid. A thorough

understanding of the interactions occurring between these three elements is necessary for biological control to realize its full potential within a given system. The interactions most easily explained are those of plant-pest and pest-parasitoid combinations. The plant on which a herbivorous insect feeds may be the only resource available to it as an individual, especially if it is in a larval stage and is unable to migrate to another host. The quality of the plant as a host impacts strongly upon the fitness, fecundity, longevity, size and development rate of herbivorous insects (Awmack & Leather, 2002; Minkenberg & Ottenheim, 1990; Musundire, Chabi-Olaye, & Krüger, 2012; Parrella, Robb, & Bethke, 1983; Urrutia *et al.* 2007). Plant quality from the point of view of a herbivorous insect can vary in the three ways: firstly, through the plant's nutritional value (Thompson, 1988), secondly, through the plant's ability to defend itself (Awmack & Leather, 2002), and thirdly, by the extent to which the plant provides an enemy free space (Gratton & Welter, 1998; Stamp, 2001). Similarly, for a developing parasitoid, the insect host will be the only source of nutrition available, so the quality of the herbivore will determine the fitness of the parasitoid (Brodeur & Boivin, 2004; Thompson, 1999; Urrutia *et al*., 2007). From these two sets of interactions it is clear that there is an indirect interaction between plant and parasitoid, whereby the plant affects the pest which in turn affects the parasitoid (Sznajder & Harvey, 2003).

There are also direct interactions between plants and parasitoids. For ectoparasitoids, developing outside the body of their host, such as *D. isaea*, the plant will provide the environment in which the larval parasitoid will develop (Malais & Ravensberg, 2003). Plants can also interact with adult parasitoids, influencing the parasitoids' ability to detect its insect host. Host detection by parasitoids can be mediated by herbivore damage to plants through the production of chemical compounds (semiochemicals), known as Herbivore Induced Plant

Volatiles (HIPVs), which parasitoids can recognise and track to infested plants (Finidori-Logli *et al.*, 1996; Takabayashi & Dicke, 1996). HIPVs vary according to the species of plant under attack (Turlings *et al.*, 1993) and the species of insect herbivore attacking it (Wei et al., 2006), thus allowing parasitoids to locate their hosts (Turlings *et al.*, 1993).

Anecdotal reports from commercial plant growers in Europe suggest that the level of suppression of the leaf mining pest *L. bryoniae* by the biological control agent *D. isaea* varied according to the host plant in which the larval leaf miners were feeding (pers. com. J. Klapwijk). This chapter describes a series of experiments designed to assess, firstly, the levels to which this variation occurs, and secondly, the nature of the interactions between plant and parasitoid. These interactions are examined in three host plant species: tomato, gerbera and French bean, the former two of which represent economically important vegetable and ornamental crops, respectively. Both of these crops are known to suffer from leaf miner infestations, though *D. isaea* is reputedly an effective control agent in gerbera but not in tomato; and the latter is the plant species in which *D. isaea* is reared commercially for use in biological control.

3.3. Materials and Methods

3.3.1. Insect culture

All insect material used in this series of experiment was derived from the laboratory populations maintained at the University of Birmingham, see Chapter 2: Insect Culture.

3.3.2. Plant material

Three species of plants were used as potential host plants during this series of experiments: tomato, *Solanum lycopersicon* L. (also known as *Lycopersicon esculentum* Mill.), var. Elanto rz; gerbera, *Gerbera jamesonii* Bolus ex. Hooker f., var. Festival; and French bean, *Phaseolus vulgaris* L., var. Tendergreen. The plants used were grown under identical conditions at the University of Birmingham's Elms Road Plant Laboratory and/or Winterbourne Gardens. Throughout each experiment plants were maintained and watered *ad libitum*.

The three plant species used in these experiments have markedly different leaf sizes and shapes, each of which as individual plants, provides a different leaf area for oviposition and larval development. The leaf area of tomato, gerbera and French bean plants was calculated using the ImageJ software package (Version 1.46, U. S. National Institutes of Health). From these measurements, to the nearest whole plant, a ratio of 2 tomato:2 bean:1 gerbera was identified as providing an equivalent leaf area for leaf miner oviposition and larval development using tomato and French bean plants approximately 20-25cm tall and gerbera plants 10-15cm tall; this ratio was used throughout this series of experiments.

3.3.3. Environmental Conditions

The first two experiments in this series were carried out under glasshouse conditions at the Elms Road Plant Laboratory, University of Birmingham, whilst the third and fourth experiments were conducted in controlled environment rooms in the School of Biosciences, University of Brimingham. As a result, the conditions under which these experiments occurred varied slightly as summarised below:

3.3.3.1. Glasshouse Conditions

The glasshouse faculty at Elms Road represents a smaller version of a commercial plant growing facility. The temperature was set at 23°C, though it varied up to 31.4°C and down to 8.3°C through the course of the two experiments. Supplementary lighting was provided for 16 hours a day by high density sodium lamps (SON-T AGRO 400, PHILIPS), providing a light intensity of 10.15 x 10 µmol.m^2.s^2.

3.3.3.2. Controlled Environment Room Conditions

The controlled environment room consisted of a single room maintained at a mean temperature of 21.4°C, although variations in temperature did occur ranging up to 33.9°C and down to 18.9°C. No natural light entered the room; illumination was provided by banks of fluorescent tubes (F58W/835, POLYLUX XLR) on a long day cycle of 16 hours light: 8 hours dark at a light intensity of 2.33 x 10 µmol.m^2.s^2.

3.3.4. Interactions between *Liriomyza bryoniae* and *Diglyphus isaea* in gerbera, tomato and French bean

In a glasshouse with a constant temperature of 23°C 10 pairs of adult *L. bryoniae* were placed into cages (BugDorm-2120, Megaview Science) arranged in a Latin square formation and containing either two gerbera, four tomato or four bean plants, with four replicates of each host plant per treatment; numbers of plants differed so as to provide a similar leaf area for oviposition (see 3.3.2). The leaf miners were allowed to feed, mate and oviposit for 48 h, after which they were removed using an aspirator. The plants were then maintained whilst mines developed in their leaves until the 8th day when half the plants were removed from each cage and placed into separate cages to act as a control. Two pairs of 1 day post eclosion

D. isaea were then added to the 'experimental' cages, and removed after a further 3 days. By the 13th day all the leaf miner larvae had emerged from the plants and pupated in both the experimental and control cages; the pupae were collected and counted. On the 19th day, the new generation leaf miner adults started to emerge from pupae which were collected, counted and sexed. On the 23rd day the new generation of *D. isaea* started to emerge and they were also collected, counted and sexed.

3.3.5. Effect of extended oviposition periods on the interactions between *Liriomyza bryoniae* and *Diglyphus isaea* in gerbera, tomato and French bean

The design of this experiment took into account the results from the previous experiment, with the aim of creating a more 'natural system' and removing some of the manipulations previously used; e.g. limited period for oviposition to produce synchronous cohorts and collection of pupae. The experiment was set up in a glasshouse with a constant temperature of 23°C, 30 *L. bryoniae* pupae, formed 8 days previously, were placed into cages (BugDorm-2120 Megaview Science) containing either two gerbera, four tomato or four bean plants, with four replicates of each host plant treatment. Between 17 and 24 adult *L. bryoniae* successfully eclosed in each cage; the mean number of adults per cage was 21.6. The adult leaf miners were allowed to feed, mate and oviposit, for the whole of their natural lives. The plants were then maintained whilst mines developed in their leaves until the 13th day when half the plants were removed from each cage and placed into separate cages to act as a control. Two pairs of *D. isaea* were then added to each experimental cage, and left to host feed, mate and oviposit for 13 days or the whole of their natural life, which ever was shorter. Paper collars were fitted around the stem of each plant to direct *L. bryoniae* larvae which had emerged from their host plant on to damp felt matting (Figure 3-1). The pupae were not

collected or counted so as to avoid damaging them and affecting their chance of survival to adult emergence. On the 23rd day the new generation of *L. bryoniae* adults started to emerge from pupae and these were collected using an aspirator. The collected *L. bryoniae* were counted and sexed under a microscope using transmitted light; morphological characteristics were used to separate the genders (see Minkenberg & van Lenteren, 1986). On the 29th day the new generation of *D. isaea* started to emerge and they were also collected, counted and sexed again using morphological characteristics to separate the genders (see Askew, 1968).

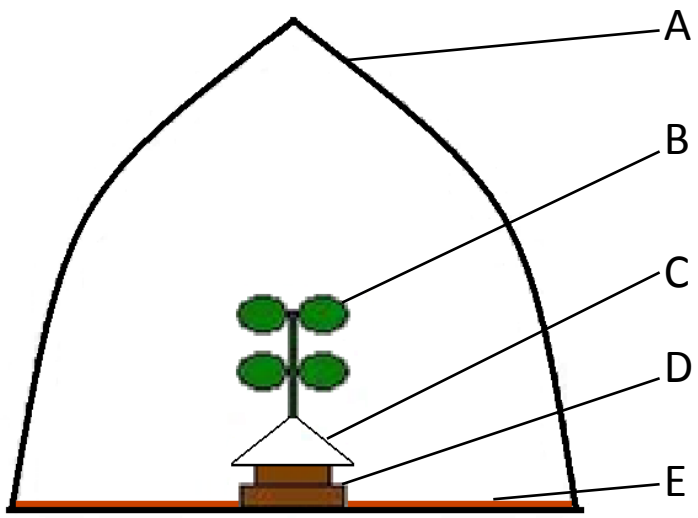

Figure 3-1 Design of the experimental system: A) BugDorm insect cage; B) *L. bryoniae* infested plant; C) paper collar; D) plant pot and tray; E) felt matting.

3.3.6. Interactions between *Liriomyza* bryoniae and *Diglyphus isaea* in tomato and French bean

This experiment was designed on the basis of the results of the two preceding experiments (sections 3.3.4 and 3.3.5) that examined the effects of the host plant on the third tropic level

i.e. the parasitoids *D. isaea*. Major changes between the method used in this experiment and those utilised before were that this experiment focused only on tomato and French bean as hosts (gerbera has been excluded), and was carried out in a controlled environment room rather than in the glasshouse used previously. Four groups of four tomato plants and four groups of four French bean plants were placed into separate cages in the controlled environment room. Forty *L. brypniae* pupae, formed 9 days previously, were placed into each cage; adults started to emerge within one day. 20-30 *L. bryoniae* adults emerged into each cage and these were allowed to oviposit onto the plants in their cages for the whole of their natural lives or until the 10th day, which ever was shorter. On the 10th day, two plants were removed from each cage and these became control plants; two *D. isaea* males and one female were added to the remaining plants. On the 13th day, one control plant from each group was dissected so that the number of *L. bryoniae* larva could be counted. At this time, *L. bryoniae* larvae started to emerge from the leaves of the remaining plants in order to pupate, and as described in section 3.3.5, a paper collar was fitted to the stem of each plant to direct larvae onto the base of the cage. These were collected, counted and then stored on damp tissue until the adults emerged. After being allowed to oviposit for 10 days, any *D. isaea* still alive were removed. At this time, one plant was removed from each of the experimental cages and *L. bryoniae* larvae were dissected out of their mines under a microscope using transmitted light so that parasitized and host-fed *L. bryoniae* larvae could be counted; these two classes of dead larvae are easily recognisable from their appearance (Figure 3-2). Remaining plants were maintained until adult *L. bryoniae* and *D. isaea* emerged and these were collected, counted and sexed.

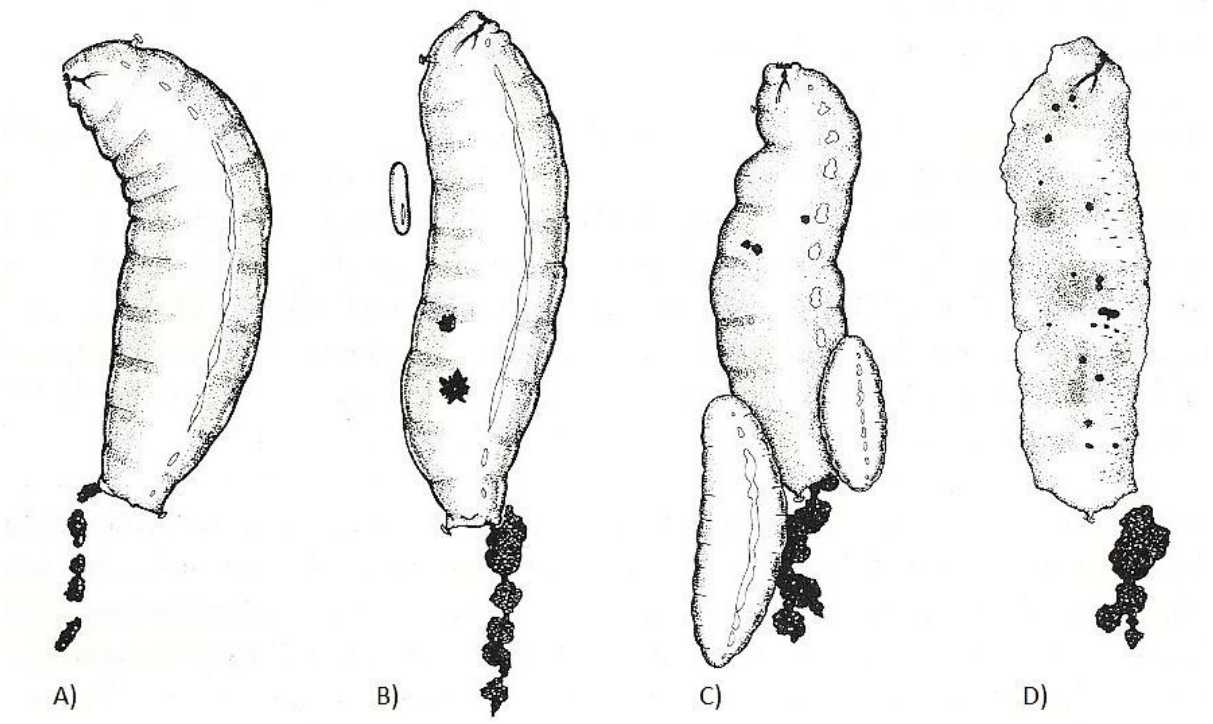

Figure 3-2: Third instar leaf miner (*Liriomyza spp.*) larvae, under different conditions: A) healthy leaf miner larva, B) turgid paralysed leaf miner larva with a *D. isaea* egg beside it, C) leaf miner larva with a few stings (for paralysation) with *D. isaea* larvae feeding on it, and, D) a dead leaf miner larvae after host feeding by adult *D. isaea* with many black spots characteristic of stings (Minkenberg, 1989).

3.3.7. Effect of earlier introduction of the parasitoid on interactions between *Liriomyza bryoniae* and *Diglyphus isaea* in tomato and French bean

This experiment largely repeated the methodology used in the preceding experiment (section 3.3.6) the exception being that newly eclosed *D. isaea* were added as soon as *L. bryoniae* mines became visible, as opposed to waiting until the leaf miner larvae were in the third larval instar. In this way *D. isaea* had passed through the preoviposition period before

even the most precocious leaf miner larvae were ready to pupate. Four groups of four tomato plants and four groups of four French bean plants were placed into separate cages in the controlled environment room. Forty *L. bryoniae* pupae were placed into each cage; adults started to eclose within one day. 20-30 *L. bryoniae* adults emerged into each cage and these were allowed to oviposit onto the plants in their cages for the whole of their natural lives or until the 7th day which ever was the shorter. On the 7th day, two plants were removed from each cage and these became control plants; two *D. isaea* males and one female were added to the remaining plants. On the 10th day, one control plant from each group was dissected so that the number of *L. bryoniae* larva could be counted. After being allowed to oviposit for 6 days, on the 13th day, any *D. isaea* still alive were removed. At this time, one plant was removed from each of the experimental cages and dissected so that parasitized and host-fed *L. bryoniae* larvae could be counted. By the 18th day all *L. bryoniae* larvae had emerged and pupated; pupae were collected and counted. The pupae were also photographed using a digital camera (Infinity 1 – 1: Lumenera Scientific, Canada) with a macro lens (Computar MLH-10X, CBC Corp., New York, NY). The images were captured as JPEGs via Studio Capture (Studio Capture DT, Studio86Designs, U.K.) and analysed using the ImageJ software package (Version 1.46, U. S. National Institutes of Health) to make an accurate measurement of the pupal length along their longest axis. Remaining plants were maintained until all adult *L. bryoniae* and *D. isaea* had emerged and these were then collected, counted, sexed, photographed and measured as described above.

3.3.8. Statistical Analysis

Statistical analysis was conducted using SPSS 20 (IMB) using One, Two or Three-Way ANOVAs or non-parametric alternatives as appropriate to test for differences between host

plants, generations and genders of insects. Figures reflect the statistical tests used with raw data, means or medians displayed as appropriate.

Log-likelihood goodness-of-fit tests were used to analyse whether the observed sex ratios differed from the expected 50:50 ratio, with Williams' correction applied to classes with less than two hundred cases (Heimpel & Lundgren, 2000; Sokal & Rohlf, 1981):

$$G = \frac{2\left(M \ln\frac{M}{0.5N} + F \ln\frac{F}{0.5N}\right)}{1 + \frac{1}{2N}}$$

Where *M* is the number of males, *F* is the number of females, *N* is *M* + *F* and the denominator is the Williams' correction factor.

3.4. Results

3.4.1. Interactions between *Liriomyza bryoniae* and *Diglyphus isaea* in gerbera, tomato and French bean

The most striking result from the first experiment was the failure of *L. bryoniae* to establish in any of the gerbera plants and as a consequence, *D. isaea* was also unable to establish in the gerbera plants. To prevent the absence of both *L. bryoniae* and *D. isaea* from gerbera cages from confounding comparisons between the other two host plant species gerbera was excluded from the statistical analyses.

3.4.1.1. L. bryoniae

By comparing only those plants in which *L. bryoniae* established it is clear that there was a disparity between plant hosts in terms of the number of *L. bryoniae* each plant species sustained from egg through to the formation of pupae (Figure 3-3). A comparison of control cages (those without *D. isaea*) showed that significantly more *L. bryoniae* pupae formed in cages of French bean plants compared with cages of tomato plants ($F_{(1,6)}$=45.753 P=0.001). In French bean cages the addition of *D. isaea* had a significant negative effect on the number of *L. bryoniae* pupae ($F_{(1,6)}$=11.127, P=0.016), however the presence of *D. isaea* has no effect on the number of pupae in cages of tomato plants.

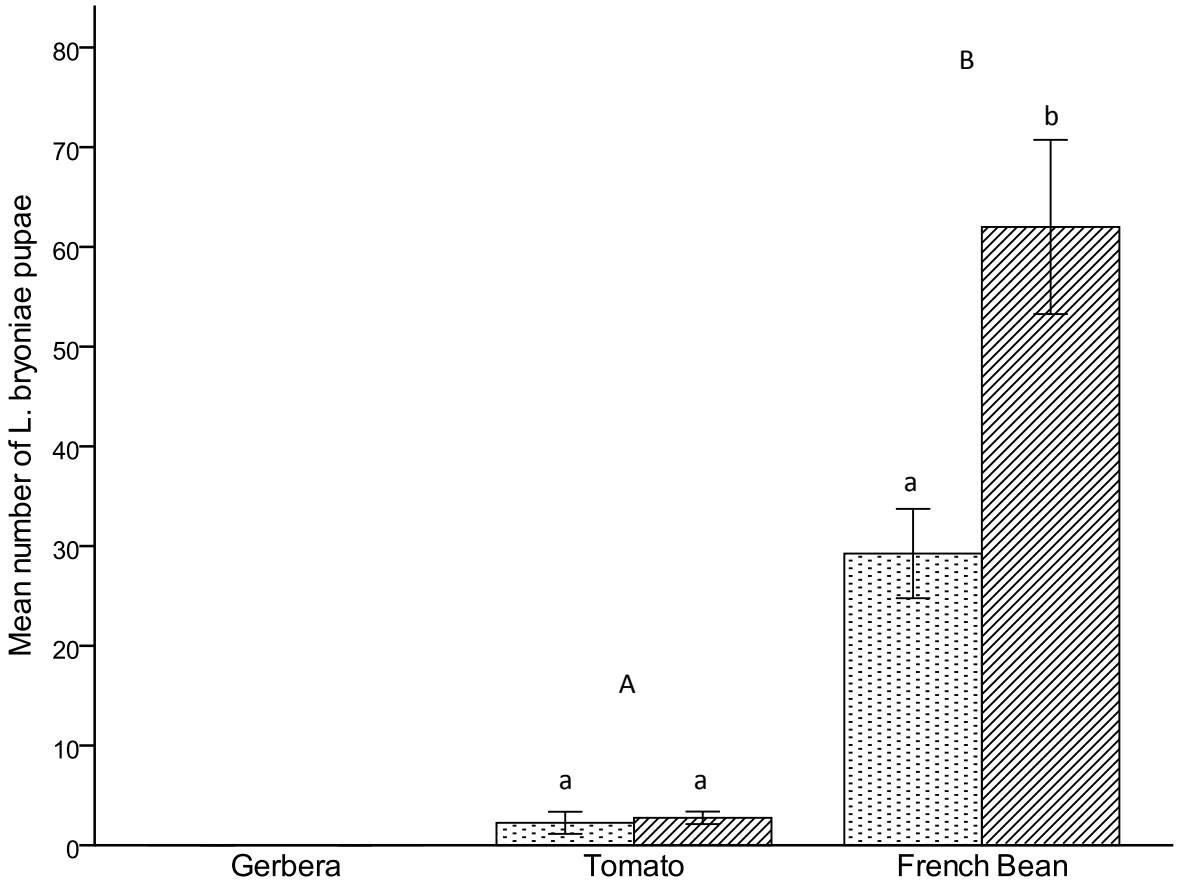

Figure 3-3: Mean ± standard error of the number of *L. bryoniae* larvae which emerged out of three different species of host plant to pupate in plants exposed to the parasitoid, *D. isaea* (dotted), and control plants, without *D. isaea* (diagonal stripe), with four replicates per treatment. Means with the same letter (uppercase for between plant species and lower case for with and without *D. isaea* within plant species) are not significantly different at P<0.05.

The number of adult *L. bryoniae* that subsequently emerged also showed a difference according to the plant host in which they had developed (Figure 3-4); however, this difference between the two species of host plant was not significant ($F_{(1,6)}$=5.861 P=0.052),

nor were the significant differences between the plants exposed to *D. isaea* and the controls.

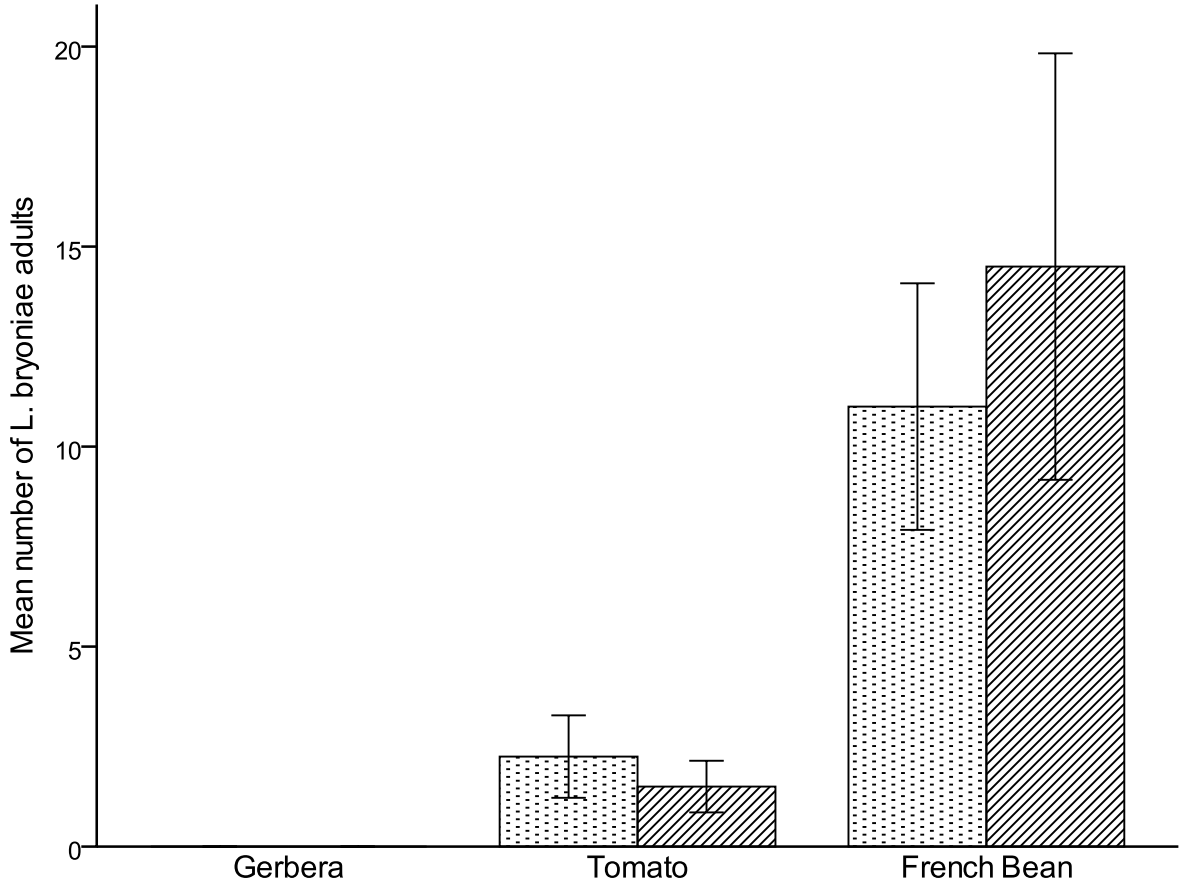

Figure 3-4: Mean ± standard error of the number of emerging *L. bryoniae* surviving to adulthood reared on three different species of hosts plants, exposed to the parasitoid *D. isaea* (dotted) and control plants, without *D. isaea* (diagonal stripes), with four replicates per treatment. At P=0.05 no significant differences were found between host plants species nor for the different treaments within host plant species.

The difference in the scale y-axis in Figure 3-3 and Figure 3-4 indicate that there is a marked decrease in number of *L. bryoniae* completing development from pupae to adult. Whilst the

absolute number of pupae and adults is greatest in the French bean populations there is variation between the two *L. bryoniae* populations in survival of those pupae collected; from cages of tomato plants 75% of *L. bryoniae* pupae successfully eclosed to become adults compared with just 28% of pupae collected from cages of French bean plants.

3.4.1.2. D. isaea

The data for *D. isaea* show two observations of interest: firstly, *D. isaea* emerged only from French bean and secondly, from across all bean replicates all but one of the emerging adults were female (Figure 3-5). A G-test with Williams correction factor showed that in the cages of French bean plants the ratio of emerging male to female *D. isaea* deviates significantly from the expected proportion of 0.5 males when significance levels are determined using χ^2 distributions at one degree of freedom ($G=20.581$, $P<0.001$).

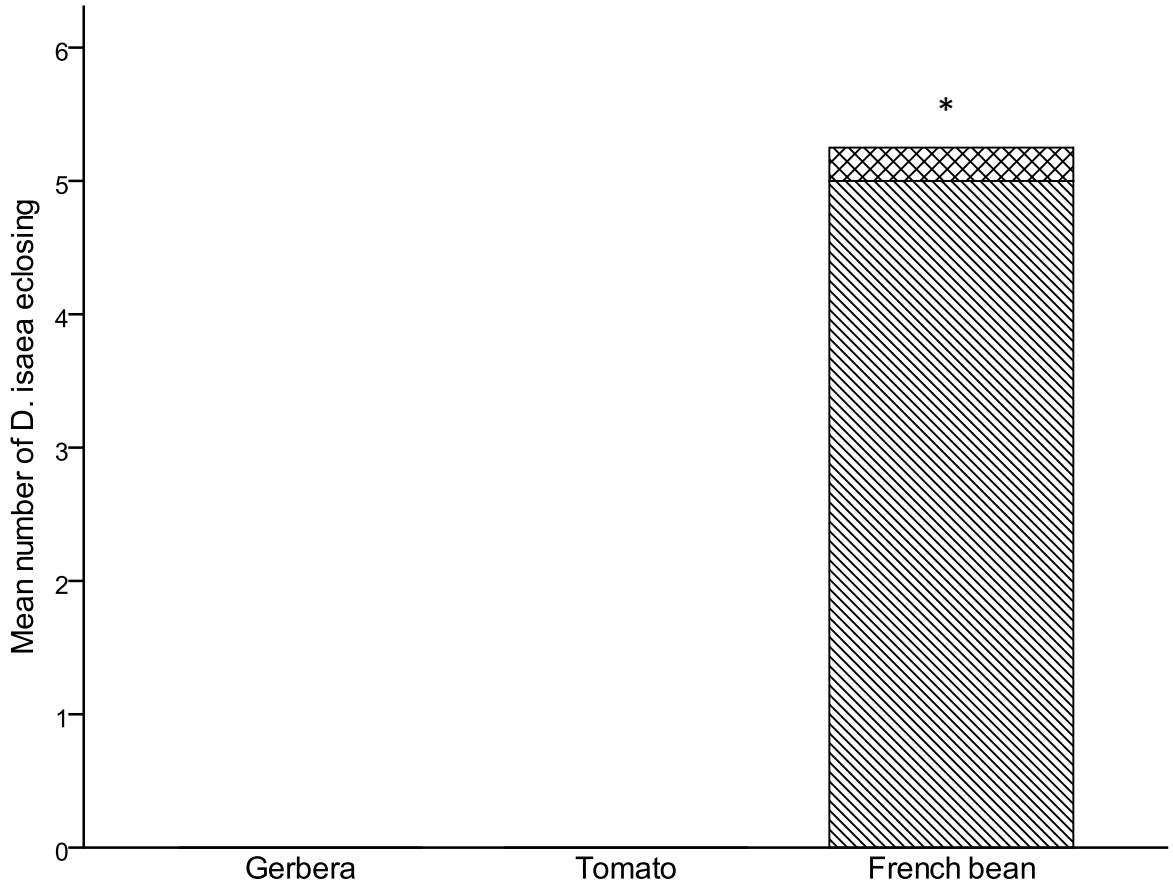

Figure 3-5: Mean number of male (checked) and female (diagonal stripe) *D. isaea* parasitoids which emerged from three different species of *L. bryoniae* infested plants, with four replicates of each plant species. Stared columns indicate a significant variation from an expected proportion of 0.5 males at P<0.001.

3.4.2. Effect of extended oviposition periods on the interactions between *Liriomyza bryoniae* and *Diglyphus isaea* in gerbera, tomato and French bean

In general, the results for this experiment are broadly the same as those for the previous experiment (detailed in section 3.4.1); again, *L. bryoniae* failed to establish in any of the gerbera plants and consequently *D. isaea* was also unable to establish in the gerbera. For this reason gerbera was again excluded from the statistical analyses.

3.4.2.1. L. bryoniae

In this experiment only adult *L. bryoniae* were collected and counted rather than the pupae. As observed in the previous experiment (Figure 3-4) tomato supported a very small population of *L. bryoniae* whilst a much larger population originated from the French bean plants (Figure 3-6). In this instance, comparison between the controls showed that the difference in the number of *L. bryoniae* adults emerging from the two species of host plant was significant ($F_{(1,6)}$=7.630, P=0.033). Contrary to the results of the previous experiment (section 3.4.1) more adult *L. bryoniae* were collected from the French bean plants which had been exposed to *D. isaea* than from the controls; however, in both French bean and the tomato, there was no significant effect of exposure to *D. isaea* in terms of the number of *L. bryoniae* adults which emerged.

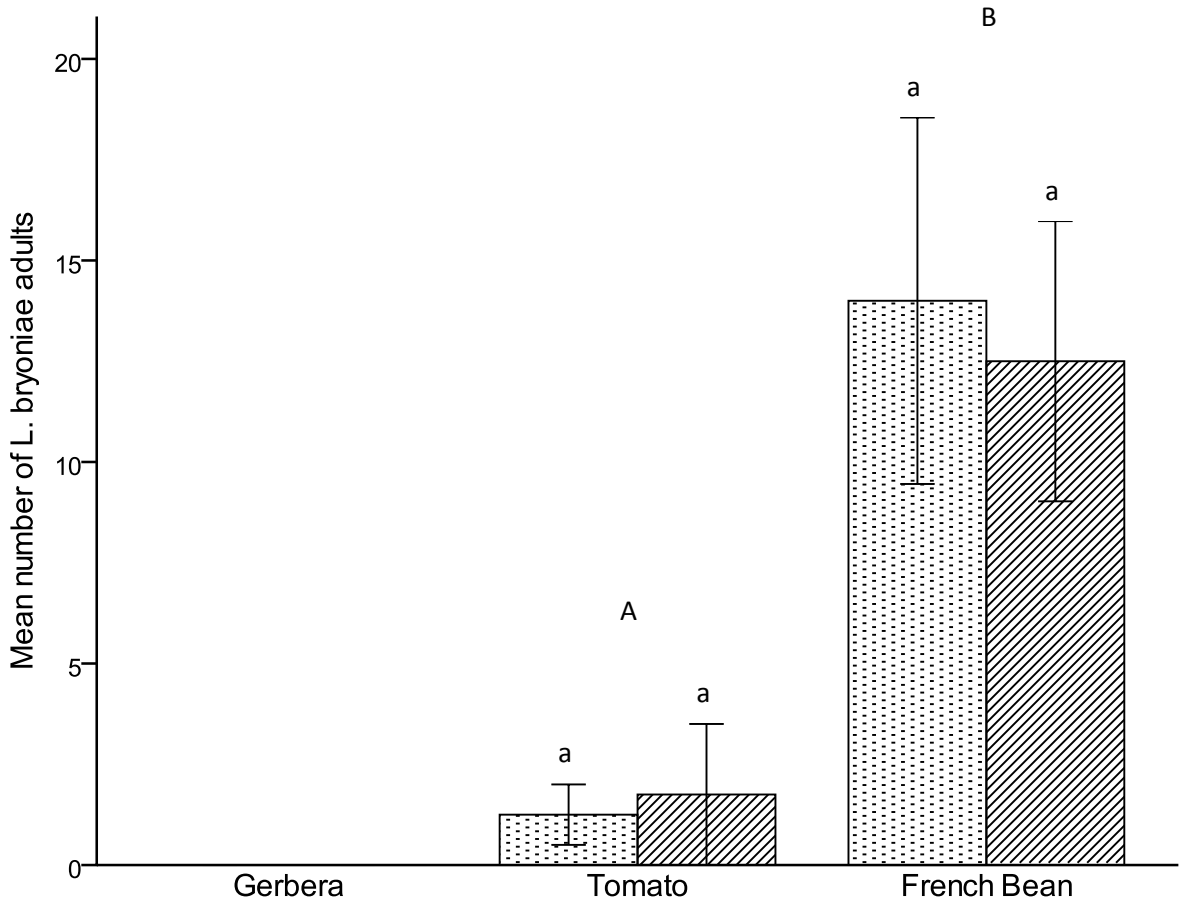

Figure 3-6: Mean ± standard error of the number of *L. bryoniae* surviving to adulthood, reared from three different species of host plant; for plants exposed to the parasitoid *D. isaea* (dotted) and control plants, without *D. isaea* (diagonal stripe), with four replicates per treatment. Means with the same letter (uppercase for between plant species and lower case for with and without *D. isaea* within plant species) are not significantly different at P<0.05.

3.4.2.2. D. isaea

The main difference between the populations of *D. isaea* emerging from this experiment and in the previous experiment is the sex ratio. In the previous experiment there was a strong female skew in the sex ratio of the emerging *D. isaea* (see Figure 3-5); however, in this

experiment, the sex ratio of *D. isaea* did not differ significantly from expected proportion of 0.5 males (G=0.243, P=0.622), albeit with a very small number of individuals (Figure 3-7).

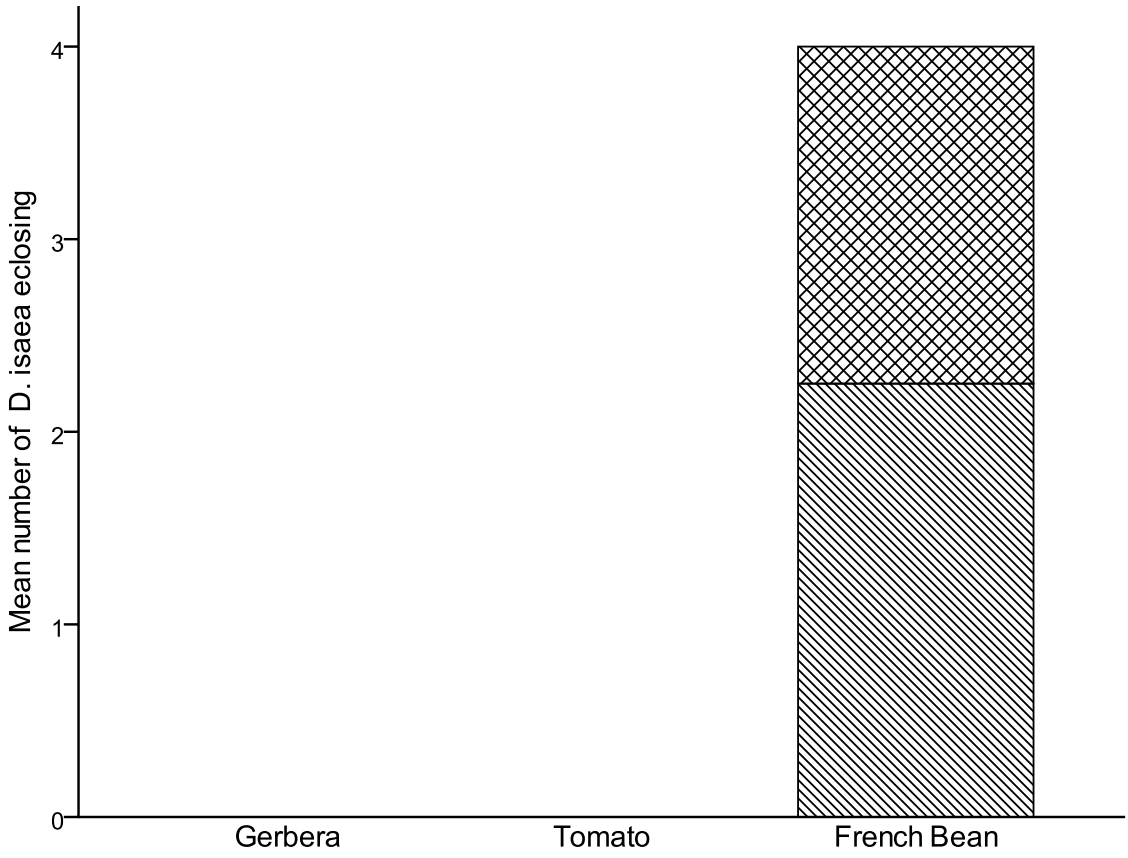

Figure 3-7: Mean number of male (hashed) and female (diagonal stripe) *D. isaea* parasitoids which emerged from three species of different *L. bryoniae* infested plant, with four replicates per plant species.

3.4.3. Interactions between *Liriomyza* bryoniae and *Diglyphus isaea* in tomato and French bean

Due to the failure of *L. bryoniae* to establish in gerbera during earlier experiments (3.4.1 and 3.4.2); gerbera was not used in further experiments.

3.4.3.1. L. bryoniae

As in previous experiments (3.4.1 and 3.4.2) there was a marked difference in the number of *L. bryoniae* supported by the two species of host plants with markedly higher densities in French bean than in tomato (Figure 3-8). This difference was significant for every life stage examined: larvae (U=0.000, Z=-2.309, P=0.021), pupae (U=0.000, Z=-2.323, P=0.020) and adult (U=0.000, Z=-2.323, P=0.020). It is also apparent from Figure 3-8 that the pre-adult life stage at which the highest mortality level occurs varied depending on the host plant; for French bean-reared leaf miners, mortality was greatest during the pupal stage with 57.6% of them failing to eclose, but for tomato-reared leaf miners, mortality was greatest during the larval stage with a 62.8% decrease between the larval and pupal stages. It is however necessary to note that the pupal densities were based on counts from different plants to those which were dissected in order to calculate the leaf miner larval densities.

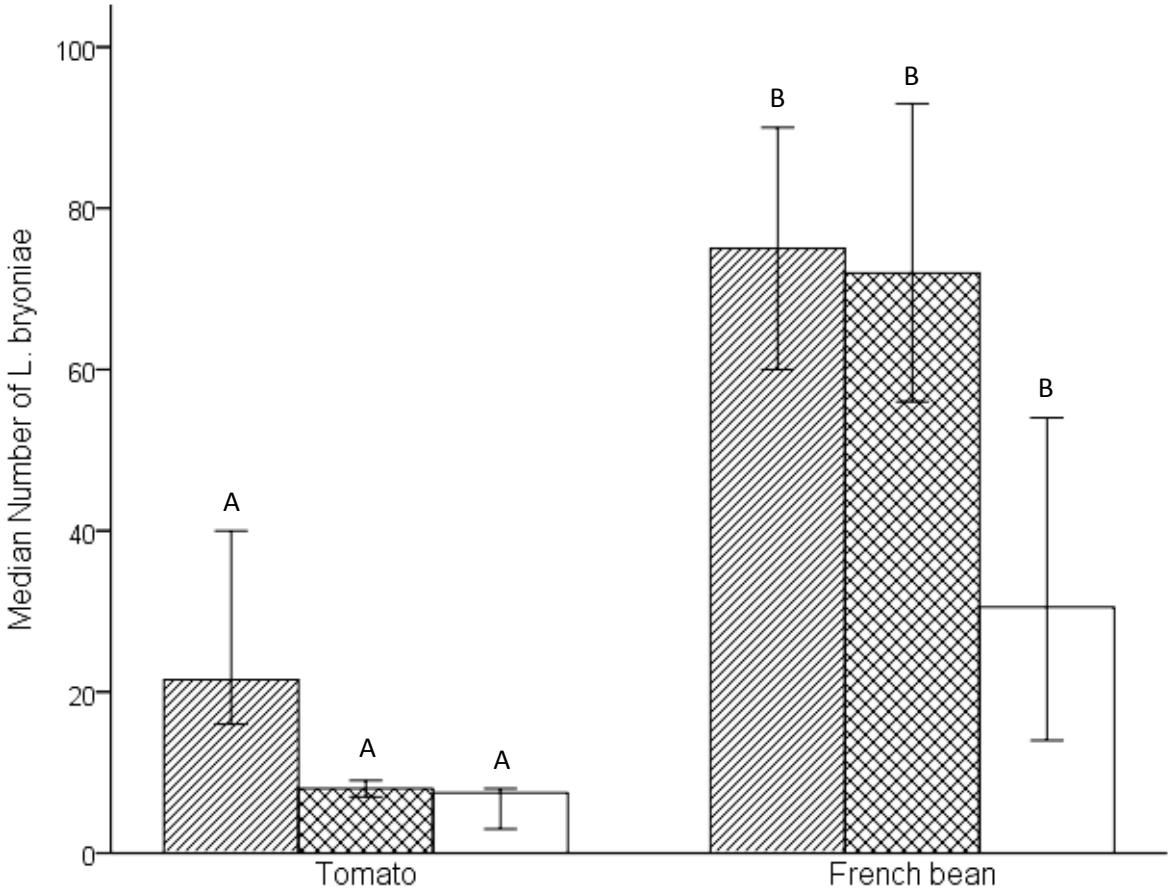

Figure 3-8: Median numbers with 95% confidence intervals of *L. bryoniae* larvae (diagonal stripe), pupae (checked) and adults (unpatterned) reared from tomato and French bean plants, with four replicates per plant species. For each *L. bryoniae* life stage, means with the same letter are not significantly different at P<0.05.

3.4.3.2. D. isaea

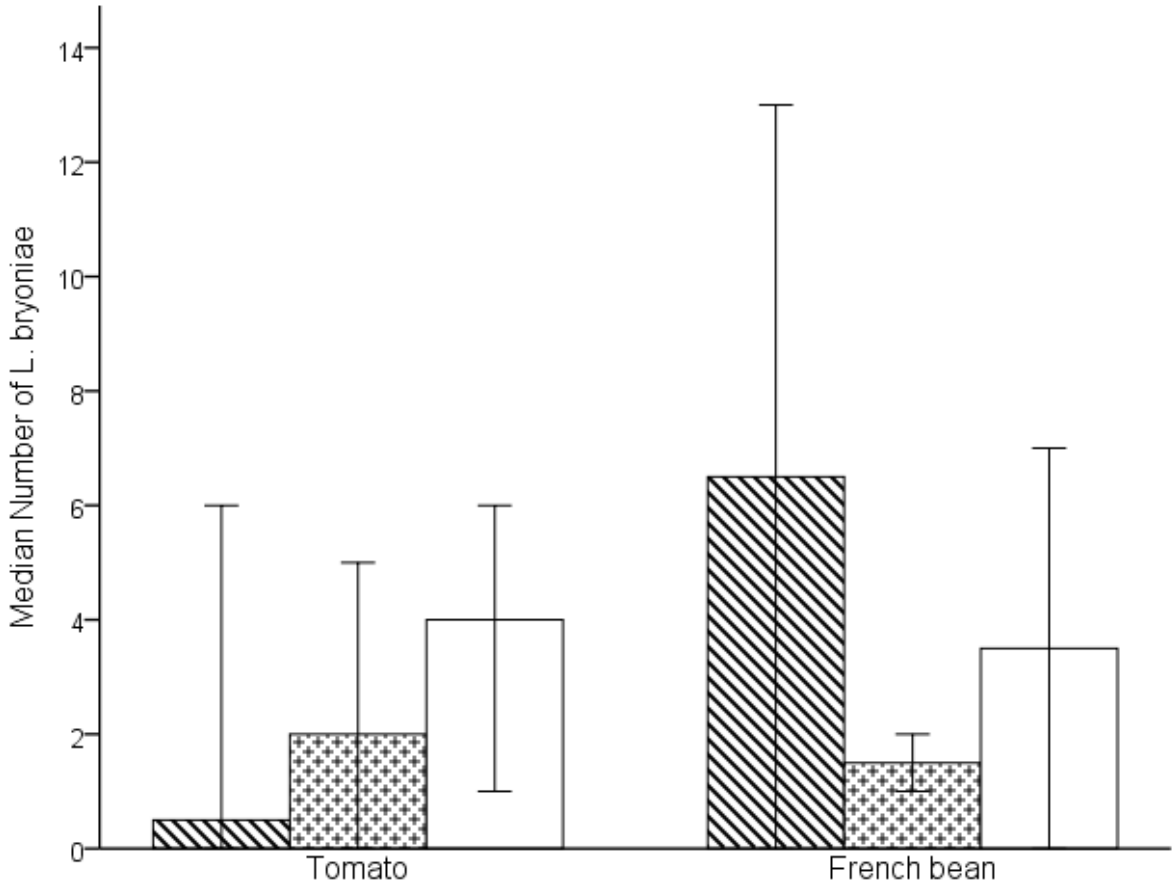

Figure 3-9: Median number ± 95% confidence intervals of *L. bryoniae* larvae in plants which had been exposed to *D. isaea* for 10 days, with four replicates per species of *L. bryoniae* infested plant. *L. bryoniae* larvae parasitized, e.g. dead with a *D. isaea* egg, larva or pupae/nymph adjacent (diagonal stripe); host fed (hashed); or other, alive or dead with no sign of parasitoid attack (unpatterned). At $P<0.05$ no significant differences were found between plant species for any class of *L. bryonaie*.

Observations from the dissection of *L. bryoniae*-infested plants exposed to *D. isaea* for 10 days showed that *D. isaea* can parasitise *L. bryoniae* larvae developing in tomato; indeed, Mann-Whitney U tests showed no significant difference in the number of parasitized *L.*

bryoniae larvae between the two host plants, nor were there any differences in other classes of larvae (Figure 3-9). However, the most important observation from this experiment is that this population of *D. isaea* was able to complete its life cycle on *L. bryoniae* larvae developing in tomato plants (Figure 3-10), which had not been observed in the two previous experiments (sections 3.4.1 & 3.4.2). The number of *D. isaea* adults which emerged from the two host plant species did not differ significantly when comparing either the total number of emergents or for the individual genders. For both the tomato reared and French bean reared *D. isaea* the sex ratio of each group did not vary significantly from a proportion of 0.5 males. It is however worth noting that generally fewer developing *D. isaea* were found in those plants which were dissected than emerged from the intact plants. Also, the female parasitoid was still alive after 10 days in three of the four cages of French bean plants whereas this was the case for only one of the four cages of tomato plants.

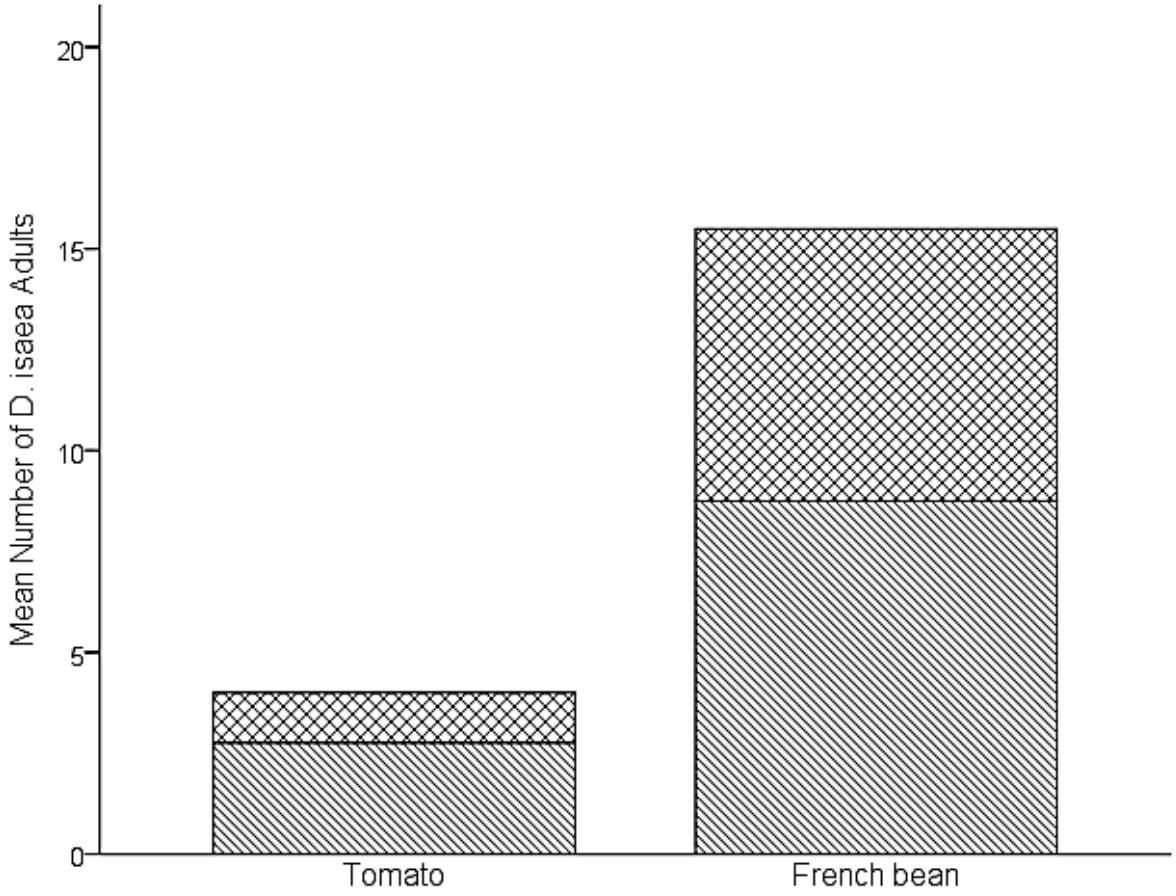

Figure 3-10: Mean number of *D. isaea* adults, males (hashed) and females (diagonal stripe), which emerged from either *L. bryoniae* infested French bean or *L. bryoniae* infested tomato plants, with four replicates per plant species. At $P<0.05$ there was no significant difference in the mean number of *D. isaea* between plant species and neither population of *D. isaea* deviated from the expected proportion of 0.5 males.

3.4.4. Effect of earlier introduction of the parasitoid on interactions between *Liriomyza bryoniae* and *Diglyphus isaea* in tomato and French bean

3.4.4.1. L. bryoniae

As with the preceding experiment, one of the interesting observations is how the relative abundance of *L. bryoniae* at each life stage varies with the host plant in which it developed. From the dissections of control plants and the collections of pupal and adult *L. bryoniae* (Figure 3-11), it is apparent that whilst tomato was the plant host in which the most larvae develop, comparatively few of these progress to forming pupae; whilst of those *L. bryoniae* pupae originating from tomato, a greater proportion became adults than of those pupae which originated from French bean. However, contrary to previous results (sections: 3.4.1, 3.4.2 and 3.4.3), there was no significant difference for any life stage in the numbers of *L. bryoniae* supported by the two species of host plant.

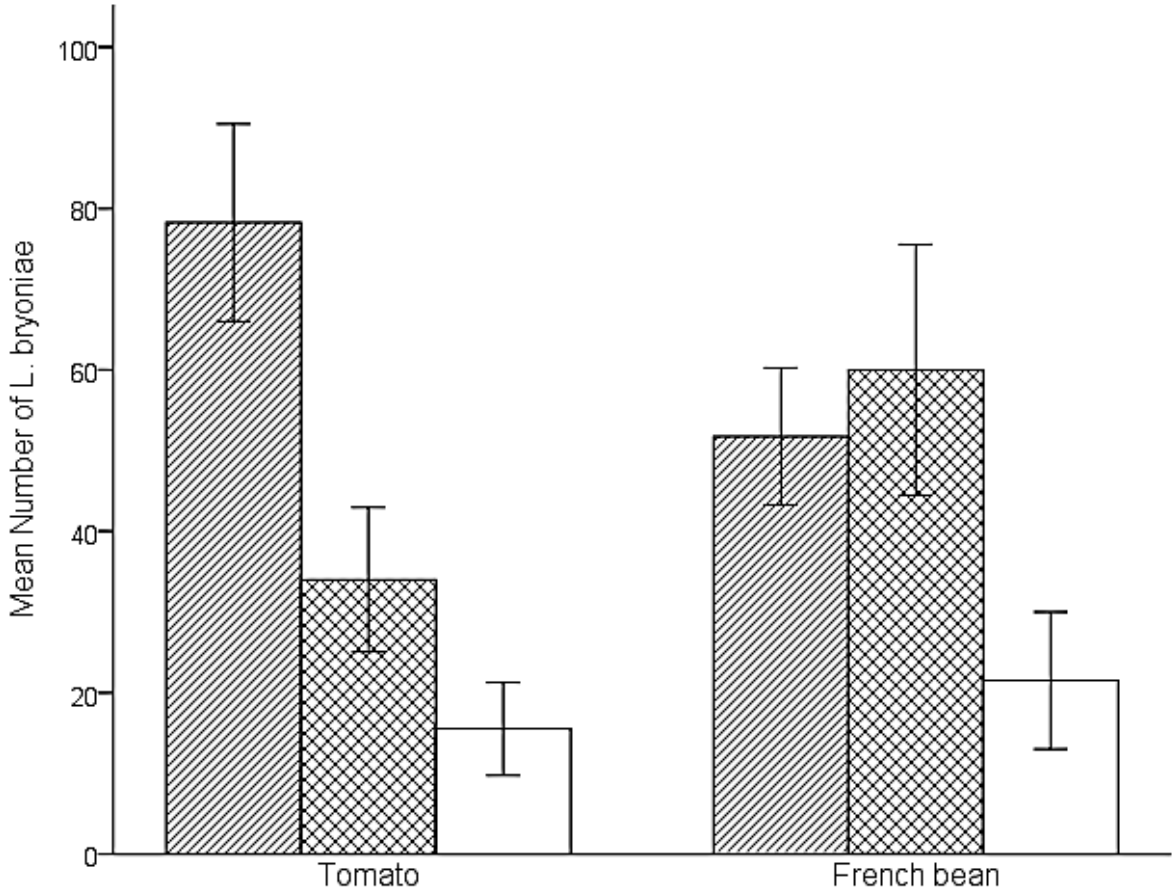

Figure 3-11: Mean ± standard error of the numbers of larvae (diagonal stripe), pupae (checked) and adults (unpatterned) of *L. bryoniae* from tomato and French bean plants, with four replicates per plant species. At $P<0.05$ no significant difference existed between host plant species for any *L. bryoniae* life stage.

3.4.4.2. D. isaea

Direct comparison with the preceding experiment (section 3.4.3) is difficult because in this experiment larvae were dissected out of their host plants on the sixth day after the addition of *D. isaea* to the cages whereas dissection previously occurred on the tenth day. However it is noticeable that where as previously there had been a greater number of parasitized *L.*

bryoniae in the French bean plants, in this experiment there were more parasitized larvae in the tomato plants (Figure 3-12), although the difference was not significant.

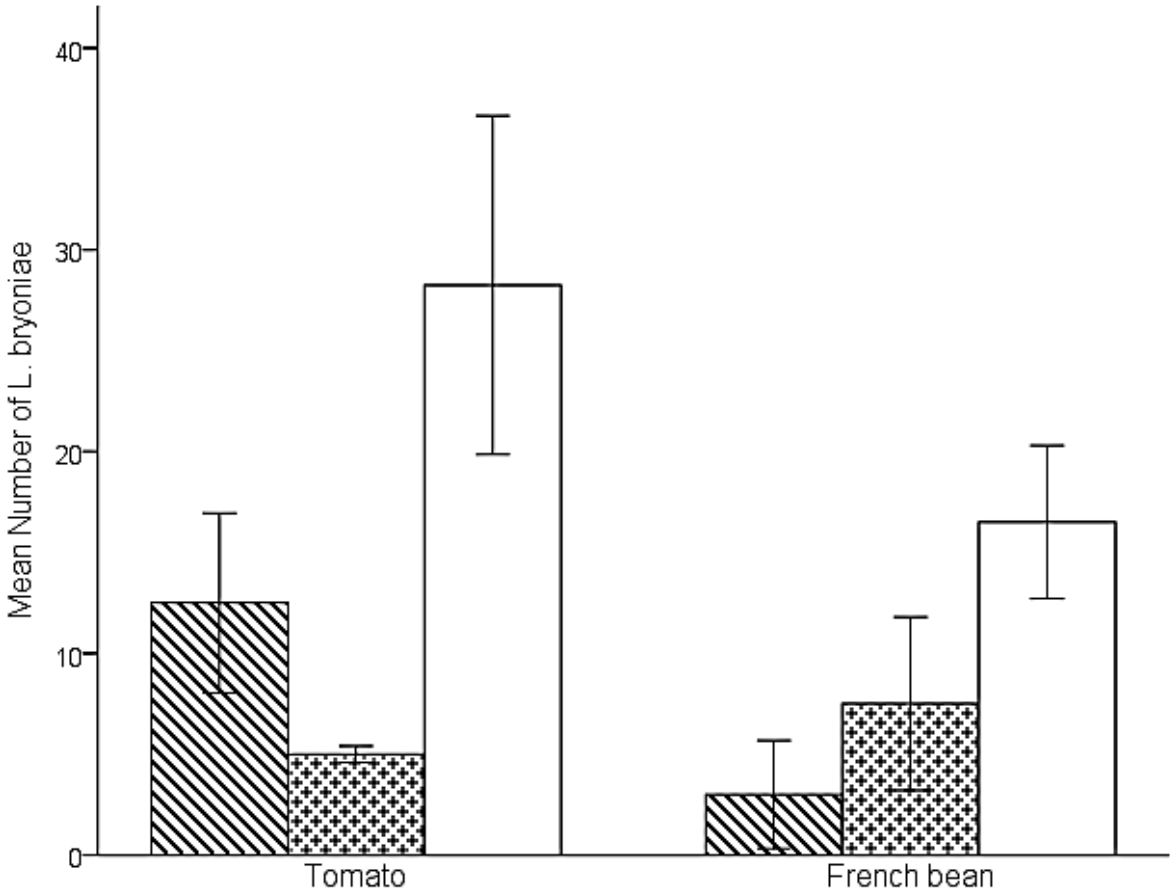

Figure 3-12: The mean ± standard error of the status of *L. bryoniae* larvae in plants which had been exposed to *D. isaea* for 6 days, with four replicates per species of *L. bryoniae* infested plant: parasitized *L. bryoniae* larvae, e.g. dead with a *D. isaea* egg, larva or pupae/nymph adjacent (diagonal stripe); host fed (hashed); or other *L. bryoniae* larvae, alive or dead with no sign of parasitoid attack (unpatterned). At $P<0.05$ no significant differences were found between plant species for any class of *L. bryonaie*.

There was no significant variation between the two host plants in terms of the number of adult *D. isaea* emerging from them (Figure 3-13). Again, for both the tomato reared and French bean reared *D. isaea* the sex ratio of each group did not vary significantly from a proportion of 0.5 males.

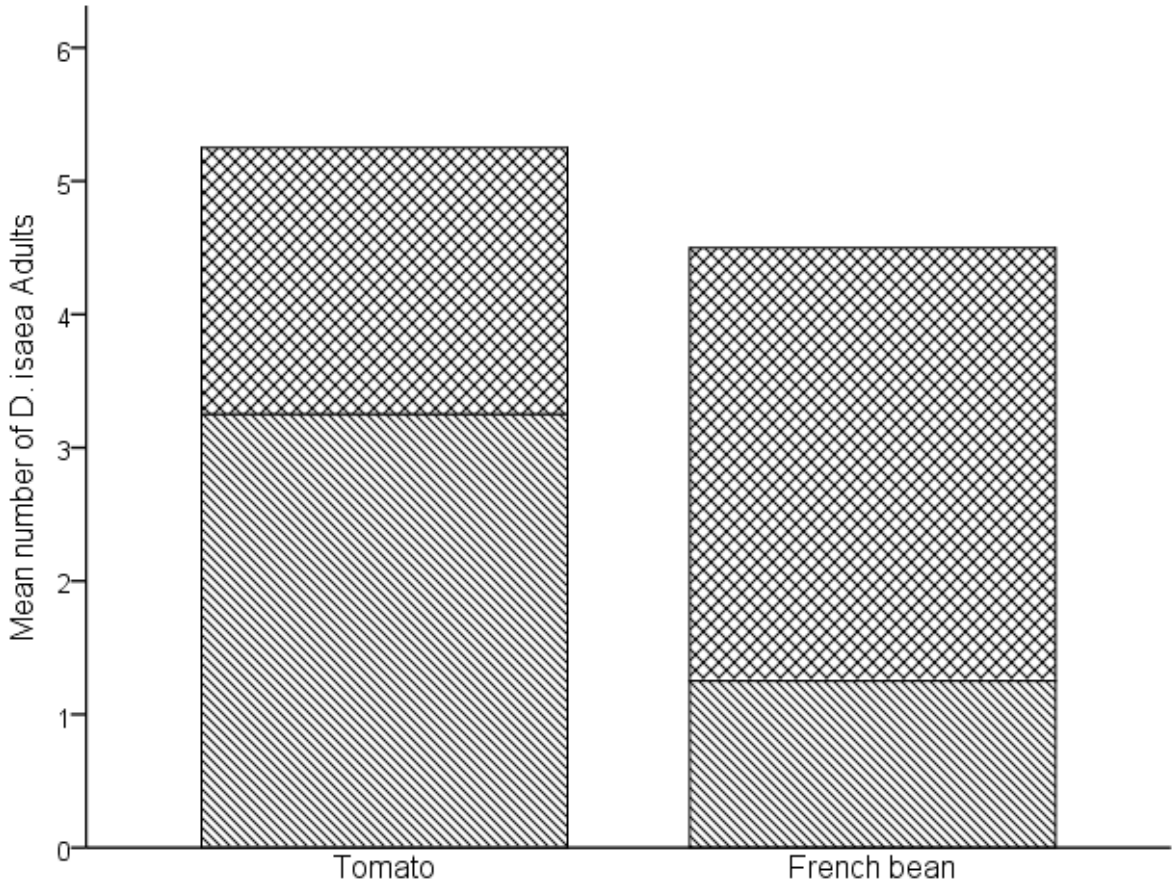

Figure 3-13: Mean number of *D. isaea* adults, male (hashed) and female (diagonal stripe), emerging from either *L. bryoniae* infested French bean or *L. bryoniae* infested tomato plants. At $P<0.05$ there was no significant difference in the mean number of *D. isaea* between plant species and neither population of *D. isaea* deviated from the expected proportion of 0.5 males.

Although the emergent *D. isaea* came from different plants to those plants dissected, if it is assumed that the mean number of parasitized *L. bryoniae* is approximately the same in both groups, a comparison of Figure 3-12 and Figure 3-13 would suggest that the majority of *D. isaea* developing in French bean emerge successfully as adults, whereas a larger proportion of those developing in tomato were unsuccessful. Finally, a comparison of Figure 3-10 and Figure 3-13 suggests that despite the earlier addition of *D. isaea*, the daily rate of parasitisation had not changed for tomato, but for French bean decreased from 1.55 to 0.75 per day.

3.4.4.3. Insect Size

Liriomyza bryoniae pupal size was significantly different between tomato and French bean ($F_{(1,365)}$=4.025, P0.046), when pupae from control cages were compared, but there was no significant difference between controls and experimental plants in either plant species (Figure 3-14).

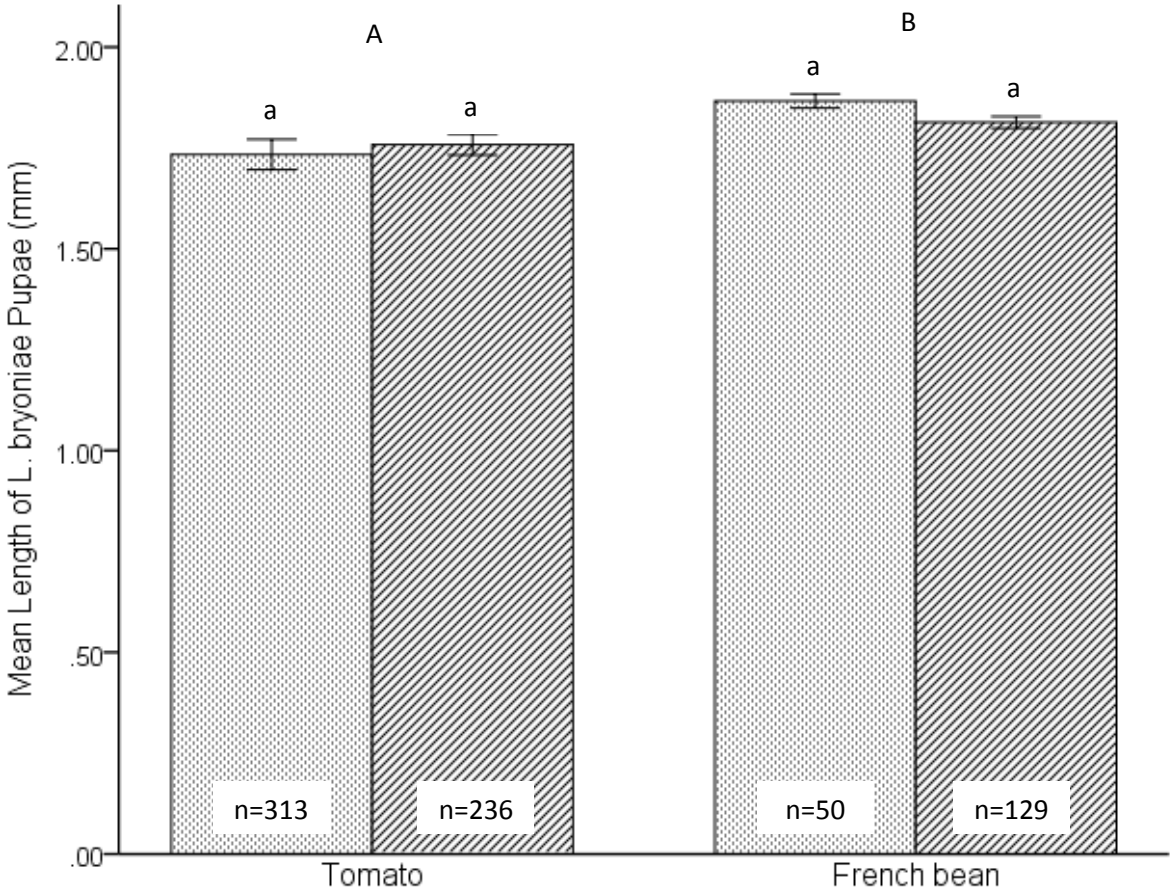

Figure 3-14: Mean length (mm) ± standard error of *L. bryoniae* pupae measured along the longest axis, pupae collected from cages of French bean and tomato, exposed to the parasitoid *D. isaea* (dotted) and controls, without *D. isaea* (diagonal stripe). Means with the same letter (uppercase for between plant species and lowercase for with and without *D. isaea* within plant species) are not significantly different at P<0.05.

A three-way ANOVA revealed that: the main effect of host plant was significant ($F_{(1,172)}$=10.348, P=0.002), with the mean wing length of French bean reared *L. bryoniae* being greater than that of tomato reared *L. bryoniae*; the main effect of gender was also significant ($F_{(1,172)}$=53.813, P<0.001), with the mean wing length of females being greater

than that of males; however, exposure to *D. isaea* (experimental cages) had no significant effect, nor were any of the interaction terms significant (Figure 3-15).

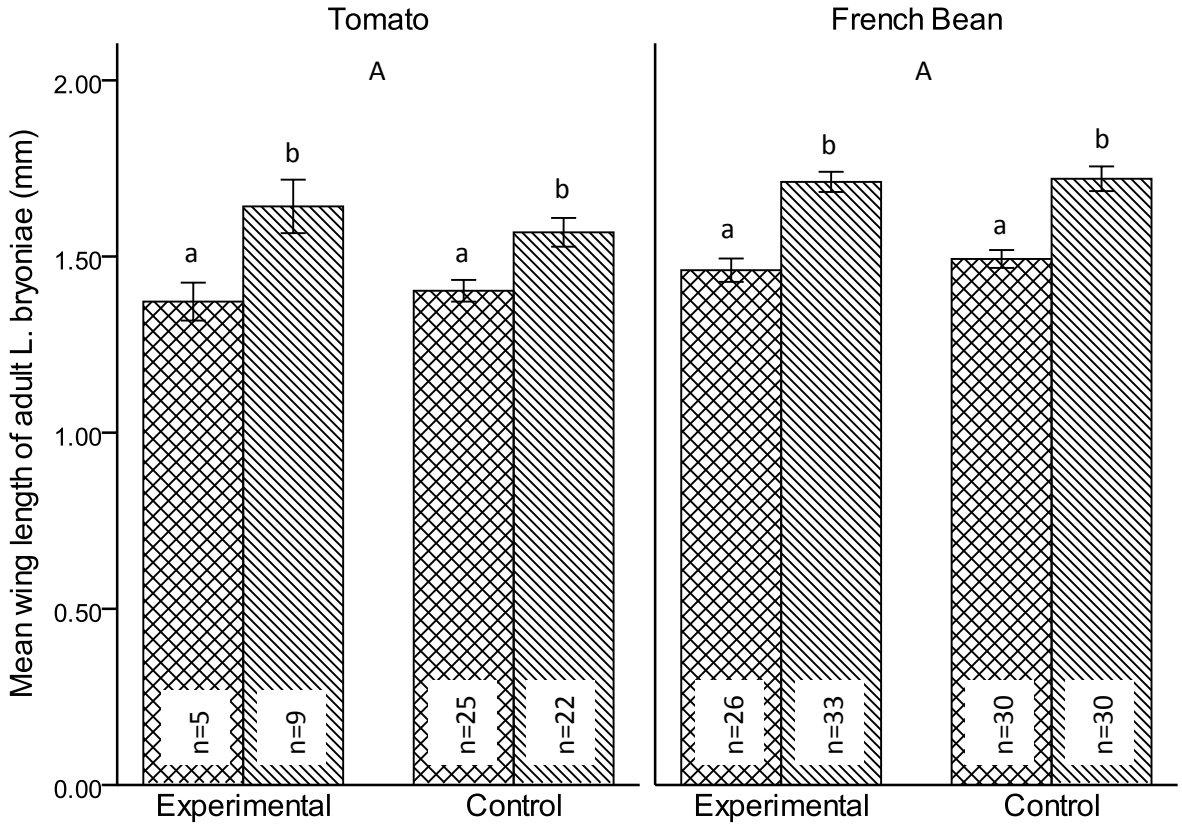

Figure 3-15: Mean wing length (mm) ± standard error of adult *L. bryoniae* reared from tomato and French bean plants, experimental cages (exposed to *D. isaea*) and control cages (without *D. isaea*) for males (hashed) and females (diagonal stripe). Means with the same letter (uppercase for between host plant species and lowercase for genders of *L. bryoniae* within host plant species) are not significantly different at $P<0.05$. No significant effect of *D. isaea* presence or absents was found.

Large standard errors in the wing length of *D. isaea* adults (Figure 3-16) reflect the small sample size of just 29 individuals from all replicates and both host plants pooled; due to the relatively large amount of variance within this sample a Two-way ANOVA suggested that neither host plant, gender or the interaction had a significant effect on wing length.

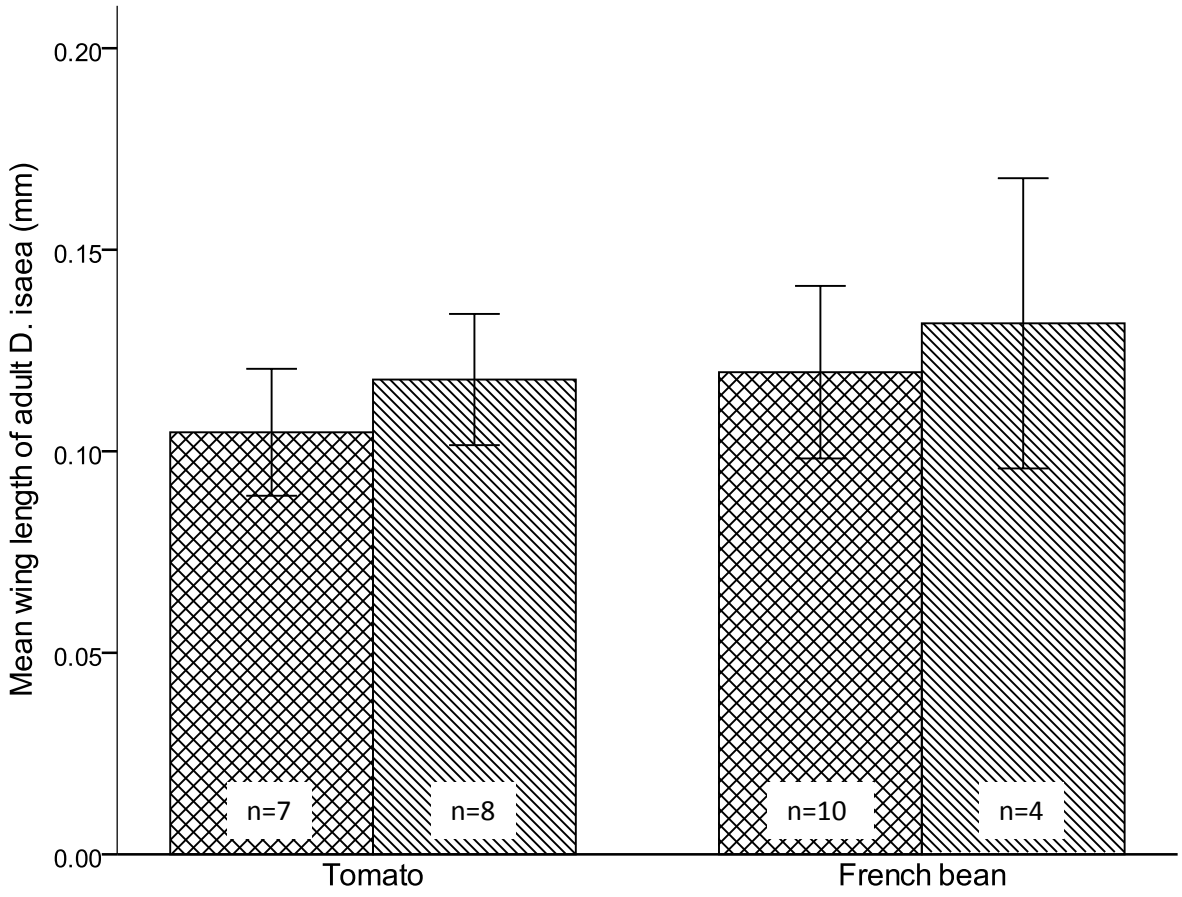

Figure 3-16: Mean for wing length (mm) ± standard error of adult *D. isaea*, males (hashed) and females (diagonal stripe), collected from cages of *L. bryoniae* infested tomato and French bean plants. At $P<0.05$ no significant difference existed between host plant species or between *D. isaea* genders.

3.5. Discussion

Liriomyza bryoniae is described as being highly polyphagous, feeding on plants from a number of different orders including economically important vegetable and ornamental species (Minkenberg & van Lenteren, 1986). Whilst anecdotal reports suggest that *L. bryoniaei* is a pest of both tomato and gerbera (pers. com. J Klapwijk), neither in this study nor in laboratory trials (unreported data) have adults of this population been observed to oviposit on the variety of gerbera used (var. Festival). A number of studies refer to the hairiness of gerbera leaves as limiting the ease with which insects can move across the under surface of the leaf (Krips *et al.*, 1999; Sütterlin & van Lenteren, 1997). However, Parrella (1987) states in his review of the *Liriomyza* that females in this genus can form leaf punctures (for either feeding or oviposition) from either the abaxial or adaxial leaf surfaces. If this is also true for *L. bryoniae* then it cannot be the case that oviposition is entirely inhibited by the hairiness of the underside of gerbera leaves. It would therefore seem likely that if there is no physical bar to *L. bryoniae* ovipositing on gerbera the population used in this study do not recognise these plants as potential hosts.

The properties possessed by a generalist herbivore species as a whole will not be consistent across all of its populations. The exact list of plant species upon which each population feeds or oviposits will vary according to the ecological, chemical, morphological and genetic factors present in a local community (Fox & Morrow, 1981). For instance, as in the current study, Caron *et al.,* (2008) found that their laboratory population of the polyphagous lepidopteron *Trichoplusia ni* failed on sweet pepper but developed successfully on both tomato and cucumber, although *T. ni* is known to feed on all three of these plants when

considered at the species level. At least two species of *Liriomyza* are known to contain cryptic species: *L. trifolii* and *L. huidobrensis* (Scheffer & Lewis, 2006; 2001) and it may therefore be that whilst the *L. bryoniae* population used in this study does not oviposit in gerbera, other populations or indeed cryptic species in the *L. bryoniae* complex can utilise this host. However, a literature search of peer reviewed material found no relevant works listing gerbera as a host of *L. bryoniae* (this search was performed using ISI Web of Knowledge (Minas; UK) with the search term 'Gerbera Liriomyza bryoniae' on the 28th of May 2012), although a number of books do list *L. bryoniae* as a pest of gerbera (Malais & Ravensberg, 2003; Onillon, 1999).

In the remaining two host plants, tomato and French bean, it is apparent that French bean is the higher quality host for *L. bryoniae* consistently supporting a greater number of immature *L. bryoniae* to pupation (Figure 3-3, Figure 3-8 & Figure 3-11). Although, neither eggs nor the ovipostion leaf punctures made by females were counted in this series of experiments, Onillon (1999) discussed how the fecundity of the related leaf miner species *L. trifolii* is also influenced by host plant. In a series of comparable studies of realised *L. trifolii* fecundity at the common rearing temperature of 15°C, female *L. trifolii* have been found to oviposit five eggs on tomato, 23 on both French bean and celery and 42 on chrysanthemum (refereces in: Onillon, 1999).

The proportion of *L. bryoniae* larvae successfully pupating out of a given host plant can vary according to the nutritional quality and chemical defences of the host plant species. The proportion of *L. bryoniae* pupating out of tomato is much smaller than the proportion which successfully pupated out of French bean; only 37% of larvae from tomato plants successfully

form pupae compared with 96% of larvae from French bean (Figure 3-8). Aside from the fact that there was a considerable difference in the number of larvae, the proportional difference in the survival provides further evidence that, for this population of *L. bryoniae*, French bean is a more suitable host.

Awmack and Leather (2002) reviewed the effects of host plant quality on herbivorous insects and concluded that the relative levels of nitrogen, carbohydrates, lipids and minerals can all affect fecundity. However, they added the caveat that variation in the effects of these nutrients will arise through interactions between the nutritional components of an insect's diet and environmental factors. Increased nitrogen content in tomato plants has been shown to increase both adult fecundity and larval survivorship in *L. trifolii* (Minkenberg & Fredrix, 1989; Minkenberg & Ottenheim, 1990). Although nitrogen content is variable between plants of the same species, French bean has been shown to have a nitrogen content of approximately 5.50%, compared with 5.00% in tomato and 2.50% in gerbera (www.weather.nmsu.edu/hydrology/wastewater/plant-nitrogen-content.htm on 13/6/12). French bean may therefore be a nutritionally superior host, encouraging both greater oviposition by adult females and higher survival from larvae to pupae.

Alternatively, plant host quality can be reduced from the point of view of a herbivore by the presence of defensive compounds. Awmack and Leather (2002) identified four main groups of defensive compounds used by plants: nitrogenous compounds (e.g. alkaloids), cyanogenic glycosides and glucosinolates, terpenoids and phenolics (e.g. tannins). Tomato is known to contain the glycoalkaloids α-tomatine and dehydrotomatine (Barceloux, 2009; Campbell & Duffey, 1979), the former of which Caron *et al.* (2008) implicated as a possible factor

contributing to the relatively poor performance of *Trichoplusia ni* and its prasitoid *Compsilura concinnate* when reared in tomato compared cucumber. This raises a second possibility that host plant defence and not nutrition is responsible for the low *L. bryoniae* larval numbers and poor survival in tomato.

Liriomyza bryoniae survival from pupa to adult appears to be low in both tomato and French bean. Minkenberg & Helderman (1990) reported 18% mortality of *L. bryoniae* pupae at 25°C, whereas in the current study mortality in some groups was as high as 58% (Figure 3-8). It is likely that manipulation of pupae causes mortality, which would account for some of the mortality when the pupae were collected and handled. The collected pupae were stored in Petri dishes with no source of shade or moisture; as *L. bryoniae* larvae would typically burrow into the soil to pupate it is possible that storage in clear Perspex containers did not provide the optimal conditions for survival from pupation to eclosion. However, a preliminary study (results not reported) showed no significant difference in survival between groups of pupae kept dry or damp nor between those kept in the dark or in the light.

In the first two experiments of this series (3.4.1 & 3.4.2) the failure of *D. isaea* to establish in gerbera and tomato can be attributed to the lack of *L. bryoniae* larvae in these plant hosts for *D. isaea* to parasitize. The most important observation regarding *D. isaea* is therefore the change in the sex ratio of the emerging adults between these two experiments, from an extreme skew to female in the first experiment to an even ratio of males and females in the second. Minkenberg & van Lenteren (1986) report the sex ratio of *D. isaea* to be highly variable. Given that Hymenoptera possess haplo-diploid determination of sex (Flanders, 1956) there has been a great deal of interest in the possible explanations for variation in

parasitoid sex ratios, especially given that from a biological control point of view, only the females contribute to the kill rate (Chow & Heinz, 2005; Heimpel & Lundgren, 2000; Minkenberg & van Lenteren, 1986; Ode & Heinz, 2002). Charnov *et al.* (1981) showed not only that the parasitoid *Lariophagus distinguendus* produces males from smaller hosts and females from larger hosts, but that large and small are relative rather than absolute sizes, as the sex ratio of offspring produced from a size class of hosts will vary according to the other size classes of hosts available to the parental generation. This has now been shown to be true for *D. isaea* (Chow & Heinz, 2005; Ode & Heinz, 2002), further to which Ode and Heinz (2002) have shown that females base their assessment of relative host size on prior history and are most strongly influenced by more recent encounters.

The *L. bryoniae* larvae presented to the parental *D. isaea* in the first of this series of experiments (3.4.1) were in a narrow age cohort and therefore presumably also in a small size cohort, as larval size is dependent on instar and instar is dependent on age (Oatman & Michelebacher, 1958). The *L. bryoniae* were also in an advanced instar, with pupation of the most precocious larvae commencing shortly after the addition of *D. isaea*. Whilst the *L. bryoniae* larvae would all have been large in absolute terms, there would not have been a great deal of variation between them in relative terms, suggesting that female *D. isaea* must to some extent have an innate knowledge of larval size in absolute terms which it is able to use when assigning a male or female egg to a host larva. In contrast, in the second of this series of experiments (3.4.2) due to the longer *L. bryoniae* oviposition period allowed in this experiment, the *L. bryoniae* larvae were more varied in age and therefore also more variable in size. The *D. isaea* sex ratio of females to males has been seen to change from 20:1 in the first experiment (Figure 3-5) to just 1.3:1 in the second experiment (Figure 3-7), which

supports the notion that like other parsitoids, *D. isaea* are able to determine the gender of their offspring and do so on the basis of the size of the *L. bryroinae* larvae on which they oviposit. Further to which the fact that the earlier addition of *D. isaea* in the fourth experiment (3.4.4) did not produce a strongly male skewed population suggests that *D. isaea* also uses a relative rather than absolute measure of host size.

In the third and fourth of this series of experiments (3.4.3 and 3.4.4) *D. isaea* was observed to utilise *L. bryoniae* reared on tomato as a host. As with *L. bryoniae*, *D. isaea* performance in tomato is poor compared with French bean (Figure 3-10); in the third experiment the average parasitism rate was just 0.4 *L. bryoniae* larvae parasitized per day by each female in tomato compared with a rate of 1.6 in French bean. The size of the *L. bryoniae* larval population can be discarded as a limiting factor because, although the *L. bryoniae* population is smaller in tomato than French bean (Figure 3-8), there would still have been an excess of *L. bryoniae* larvae even if parasitism occurred at the same rate as in French bean. It is, however, worth considering the fact that *D. isaea* is a synovigenic species, meaning that adult females must host feed in order to meet the energetic costs of egg maturation (Giron *et al.,* 2004) and that the quality of the larval *L. bryoniae* as a source of nutrition for female *D. isaea* will be effected by the quality of the plant of which the larval *L. bryoniae* has itself been feeding. It is also known that insect herbivores can bioaccumulate plant allelochemicals (Bowers & Stamp, 1997) and that unpalatable prey can have a negative effect on the fitness of predators (Stamp, 2001). It is therefore possible that when host feeding on *L. bryoniae* larvae reared on tomato, *D. isaea* also consume and are adversely affected by α-tomatine or other toxic compounds, which have a negative effect on the rate at which they parasitise *L. bryoniae*.

The recommended rate of release of *D. isaea* in commercial glasshouses is 1 individual for every $4m^2$ (www.koppert.com) and the Bugdorm cages used in these experiments were $60cm^2$; for this reason in the third and fourth of this series of experiments (3.4.3 & 3.4.4) only one female parasitoid was released into each cage. In the fourth experiment the rates of parsitisum by *D. isaea* on *L. byroniae* were not significantly different between tomato and French bean cages, however, in two cages of French bean there was a zero rate of parasitism, suggesting that the parasitoids died before starting oviposition. However, comparison between the third and fourth experiments shows that the earlier introduction of *D. isaea* had no effect on parasitism rate in tomato, and a possible negative effect in French bean.

The adult body size of herbivorous insects is normally positively related to potential fecundity (Klingenberg & Spence, 1997) and field data from Videla *et al.* (2006) showed that the leaf miner *L. huidobrensis* was most numerous in the plant host *V. faba* in which it attained the greatest size, demonstrating the link between size and performance. Likewise, the performance of parasitoids are often correlated to their body size (West, 1996) and parasitoid body size is limited by the size and quality of their host (Urrutia *et al.,* 2007). In the final experiment of this series it was therefore interesting to look at the adult body size of both L*. bryoniae* and *D. isaea* in the offspring generation.

Both the pupal length and wing length of adult *L. bryoniae* was greater in French bean than in tomato, which is consistent with studies on other *Liriomyza* species as the size of both *L. tifolii* and *L. sativae* has been shown to vary according to their host plant (Musundire *et al.*, 2012). This finding also reinforces the established link between preference and performance

in herbivorous insects (Videla *et al.*, 2006) as *L. bryoniae* is both larger and more numerous when reared in French bean. It is however surprising that there is not a similar relationship between adult size and host plant in *D. isaea*, as it has previously been shown that parasitoid size is directly dependent on the size of their host (Salvo & Valladares, 2002, 1995). This suggests that either, there is sufficient nutrition in *L. bryoniae* larvae, regardless of host plant, for developing *D. isaea* to achieve their maximum potential size, or that the size of *D. isaea* developing on the larger *L. bryoniae* larvae in French bean is limited to a size equivalent to that of *D. isaea* developing on the smaller *L. bryoniae* larvae in tomato by some other factor.

In conclusion, the host plant on which it is reared affects *L. bryoniae* in numerous ways including: the numbers at each successive life stage (larval, pupal, adult) in the offspring generation, survival to pupation, survival from pupation to eclosion and pupal and adult size. The impacts of the host plant on larval *L. bryoniae* in turn affects the number of *D. isaea* in their offspring generation; however, there does not appear to be a 'knock on' impact on the sex ratio or size of *D. isaea*.

4. MULTIGENERATIONAL EFFECTS OF NOVEL HOST PLANTS ON A POLYPHAGUS LEAF MINER, *LIRIOMYZA BRYONIAE,* AND ITS PARASITOID, *DIGLYPHUS ISAEA*.

4.1. Abstract

Agricultural monoculture and long term laboratory culture have both been associated with the formation of host races in herbivorous pests thought to be polyphagous at the species level. Similarly, parasitoids considered as generalists at the species level may be more specialist on a local scale, further to which, there is concern that mass rearing for biological control programmes may exasperate these relationships. Previous observations of the herbivore *Liriomyza bryoniae* and its parasitoid *Diglyphus isaea* has shown that individual size and population structure of a long term bean-reared laboratory culture changes when moved from the conventional host plant and onto a novel one. This series of experiments showes that whilst long term bean-rearing does not diminish the potential of *L. bryoniae* to infest tomato, even after three generations of habituation to tomato larval mortality and individual size are still negatively affected. The habituation of *L. bryoniae* to tomato does not appear to effect *D. isaea*. Whilst habituation of *D. isaea* to tomato has no effect on offspring number or individual size, tomato-habituated *D. isaea* appear to utilise fewer *L. bryoniae* larvae for host feeding. Implications for the biological control of *L. bryoniae* by *D. isaea* are discussed.

4.2. Introduction

It is increasingly apparent that whilst many herbivorous insect species may appear to be polyphagous when considered across the whole of their geographical range, local populations of those same species are considerably more discerning in their choice of food plants (Fox & Morrow, 1981; Nagoshi & Meagher, 2008; Sword & Dopman, 1999). At its most extreme this may lead to the formation of distinct host races; groups of individuals which differ genetically from other groups due to host plant preferences causing partial reproductive isolation between such groups (Diehl & Bush, 1984; Parrella, 1987), or even speciation. Parrella (1987) asserts that agricultural monoculture is a factor likely to facilitate the development of such host races in herbivorous pests such as *Liriomyza* leaf miners; subtle changes associated with the formation of host race may affect the efficacy of pest control methods. Laboratory populations of apparently polyphagous pests are generally maintained on a single species of plant which is inexpensive or easy to grow even when the crop of concern may be an entirely different plant (Parrella *et al.*, 1983). This combined with the fact that laboratory populations are prone to genetic drift and genetic bottle necks (Powell & Wright, 1988) is a major concern for the study of agricultural pests as it may lead to the incorrect assessment of biological characters and the preconditioning of an insect to its colony host thus potentially altering the outcome of control programme efficacy assessments (Parrella *et al.*, 1983).

In the third tropic level parasitoids also show host related specialisation; for instance, *Diaeretiella rapae* at the species level is considered to be a generalist parasitoid utilising over 60 species of aphids as hosts (Antolin *et al.,* 2006), however, locally populations of *D. rapae*

in northern Colorado predominantly utilise just two host species and that they show greater survival and productivity on these hosts than on alternative hosts. Antolin *et al.*, (2006) concluded that *D. rapae* is a serial specialist with populations attacking particular host species according to both their geographic location and the time of year. Further to which Hatherly *et al.*, (2009) have demonstrated that the efficacy of the predatory mirid *Macrolophus caliginosus* as a biological control agent varies not only according to its prey species but also with the host plant on which the herbivore was feeding. Conversely, no effect of host plant was found on the population genetic structure of *Chelonus insularis* and *Camploetis sonorensis* both of which parasitise the Fall Armyworm *Spodoptera frugiperda* (Jourdie *et al.*, 2010). The study performed by Jourdie *et al.*, (2010) unlike that of Hatherly *et al.*, (2009) was performed using wild caught and not laboratory cultured insects. This mirrors the concerns previously discussed that mass reared and laboratory populations may succumb to genetic drift and genetic bottle necks causing them to become habituated to the plant-herbivore combination on which they are maintained.

The herbivorous pest *Liriomyza bryoniae* is known to be polyphagous (Minkenberg & van Lenteren, 1986), however, the population used in this series of experiments has for many years been reared exclusively on bean plants. It had previously been observed that when adults from this population of *L. bryoniae* were allowed to oviposit in the alternative plant host, tomato, although a comparable number of mines were formed, by comparison with bean, far fewer *L. bryoniae* successful survived to adulthood (Figure 3.11). Likewise, the *L. bryoniae* individuals reared on tomato were significantly smaller than those reared on bean (Figure 3.14). These observations were based on only the first generation of *L. bryoniae* to be reared in the 'novel' host tomato and as it was not clear by what mechanism these

changes, both in population structure and body size were brought about, it has been considered to be of interest to investigate whether this effect persisted over multiple generations or whether the population structure and body size reverted to that observed with bean-reared populations after habituation to tomato. Further to which, the effect that habituation of *L. bryoniae* to tomato might have on the third tropic level, e.g. the patasitoid *D. isaea,* was also unclear; the question of whether *D. isaea* is affected by the habituation of *L. bryoniae* to tomato was therefore examined. Similarly it had previously been observed that *D. isaea* populations were smaller when reared from *L. bryoniae* in tomato than in bean (Figure 3.10), although no effect of plant host had been observed on the size of *D. isaea* (Figure 3.16). Again, these data relate only to the first generation of *D. isaea* to be reared in tomato after many generations of rearing on bean, so it was unclear what effects habituation to tomato may have on *D. isaea*. These questions are important as *D. isaea* is commercially cultured on leaf miners reared in bean for use in biological control, however, these bean reared *D. isaea* are commonly used against *L. bryoniae* in the glasshouse cultivation of tomatoes (pers. com. J. Klapwijk).

4.3. Materials and Methods

4.3.1. Insect culture

All insect material used in this series of experiment was derived from the laboratory populations maintained at the University of Birmingham, see Chapter 2: Insect Culture.

4.3.2. Plant material

Three species of plants were used during this series of experiments: tomato, *Solanum lycopersicon* L. (also known as *Lycopersicon esculentum Mill.) var. Elanto rz; broad bean, Vicia faba* L. var. Sutton Dwarf; and French bean, *Phaseolus vulgaris* L. var. Tendergreen. The plants used were grown by University of Birmingham horticulturists at Winterbourne Gardens. Throughout each experiment plants were maintained and watered *ad libitum*.

The three plant species used in these experiments have markedly different leaf sizes and shapes, however, measurement of the leaf area of tomato, broad bean and French bean plants using the ImageJ software package (Version 1.46, U. S. National Institutes of Health) showed that a ratio of 1 tomato: 1 broad bean: 1 French bean would provide equivalent leaf area across the three plant species.

4.3.3. Environmental Conditions

The series of experiments described in this chapter were performed in the same controlled environment room as those in Chapter 3, described in section 3.3.3.2.

4.3.4. Multigenerational effects of *Liromyza* on a novel host

Twelve cages, six containing a tomato plant each and six with a broad bean plant, were established in controlled environment room at 20±1°C and with a long day photoperiod (16h light: 8h dark). Ten *L. bryoniae* pupae taken from the stock bean-reared culture were placed into each cage. Adult *L. bryoniae* started to emerge within 24 hour. The mines forming in the plants were counted by eye on the eighth day after adult emergence. As larvae emerged from the plants to pupate they were directed onto the base of the cage by a paper collar fitted around the plant stem; this prevented the larvae from entering the soil

and allowed for easy collection of pupae. Once all the pupae had been collected they were photographed and measured using the software package ImageJ. A preliminary study had shown that measurement of pupal size was a good proxy for adult size; Pearsons product-moment correlation indicates a significant positive association between pupal and adult body length (r=0.477, d.f.=30, P=0.008). Ten pupae (or as many as possible if fewer than ten pupae had formed in a cage) were then returned to their cage of origin to start a new generation in a fresh plant. Adults were allowed to emerge (from both the pupae returned to the cage and the remaining pupae) and were then counted. This was repeated until adult emergence in the third generation.

4.3.5. Effects of *Lirimoyza* host habituation on bean reared *D. isaea*

Cages were established containing either conventionally bean reared *L. bryoniae* pupae with French bean plants, conventionally bean reared *L. bryoniae* pupae with tomato plants or tomato habituated (four generations) *L. bryoniae* with tomato plants. Each cage contained four plants and 30 pupae, with three replicates of each plant-*L. bryoniae* combination. Once the mines of second instar *L. bryoniae* larvae became visible the number of mines in each plant was counted, paper collars where fitted to the plants for leaf miner pupae collection and three female and two male conventionally cultured *D. isaea* were added to each cage. *Liriomyza bryoniae* pupae were collected, counted and measured using the software package ImageJ. Emergent *D. isaea* were also collected and frozen before being counted and sexed; *D. isaea* wing length was also measured using ImageJ. In preliminary tests wing length has been shown to be a good proxy for total body length; Pearson's product moment correlation indicates a strong positive association between total body length and wing length (r=0.81, d.f.=29, P<0.001).

4.3.6. Effects of natal host on *D. isaea*

D. isaea were cultured for one generation on *L. bryoniae* in tomato plants after which the efficacy of tomato-reared *D. isaea* as a biological control agent of *L. bryoniae* in tomato was compared with that of conventionally bean-reared *D. isaea*. Three *D. isaea* adults (two females and one male), from either the one generation tomato-reared population or the conventionally reared stock culture, were added to cages containing three tomato plants with 72-94 second instar *L. bryoniae* per cage. There were three replicates of each treatment. A further 10 male and 10 female *D. isaea* from each group were frozen and then measured using the ImageJ software package. After *L. bryoniae* larvae emerged from the plants to pupate they were collected and counted. The new generation of *D. isaea* adults were also collected when they emerged from the plants and were then frozen before being sexed, counted and measured using the ImageJ software package.

4.3.7. Statistical Analysis

Statistical analysis was conducted using SPSS 20 (IMB) using One, Two or Three-Way ANOVAs, paired T-tests, or non-parametric alternatives, with suitable post-hoc tests as appropriate, to test for differences between host plants, generations and genders of insects. Log-likelihood goodness-of-fit tests have been used to analyse variations in sex ratios (as described in 3.3.8). Figures reflect the choice of statistical tests with raw data, means or medians displayed to best reflect the analysis used.

4.4. Results

4.4.1. Multigenerational effects of *Liromyza* on a novel host

4.4.1.1. Life stages and population level effects

In generation zero (the pupae originally taken from the stock culture), six replicate populations of 10 *L. bryoniae* pupae were added to cages containing a broad bean plant and a further six replicates were added to cages containing a tomato plant. In every case generation one larvae formed mines in the leaves of the plants; larvae emerged to form pupae and became adults in varying numbers (Figure 4-1). When Mann-Whiteny U tests were used to compare the number of leaf miners in each life stage on the two host plants there was no significant difference between the number of mines on bean and tomato (U=17.5, Z=-0.08, P=0.936); however, there was a significant difference in the number of pupae formed (U=2.0, Z=-2.567, P=0.010) and number of adult flies emerging (U=5.5, Z=-2.005, P=0.045), both of which were higher in bean (Figure 4-1).

At the end of generation one too few adults had emerged in three of tomato replicates and one of the bean replicates for a second generation to be established from these populations. In all the remaining populations a second generation of larvae formed mines, became pupae and adults emerged. As with generation one there was no significant difference between the number of mines on bean and tomato (U=6.0, Z=-0.447, P=0.655); however, there was a significant difference in the number of pupae formed (U=0.000, Z=-2.236, P=0.025) and number of adult flies emerging (U=0.000, Z=-2.236, P=0.025), both of which were higher in bean (Figure 4-1).

Between generations two and three one further population of *L. bryoniae* on bean went extinct however no more tomato population went extinct. As with generations one and two there is no significant difference in the number of mines between plant hosts (U=2.500, Z=-1.260, P=0.208) and whilst there was significant difference in survival to pupation (U=0.000, Z=-2.141, P=0.032) there was, unlike in previous generations, no significant difference between host plants for the number of adult flies emerging (U=1.000, Z=-1.834, P=0.067).

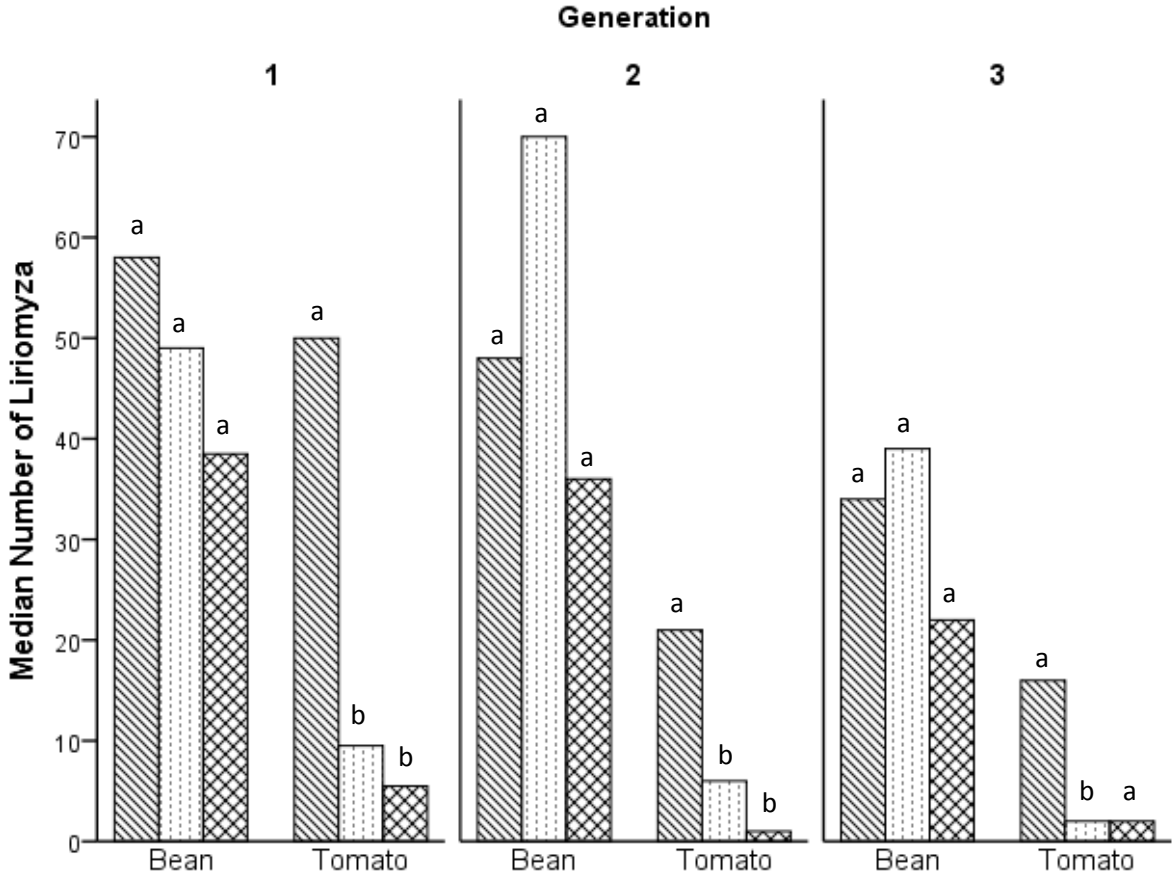

Figure 4-1: Median number of *L. bryoniae* in each life stage (larva: diagonally striped, pupa: dotted, adult: hatched) in the two host plants, bean and tomato, across generations one to three, initially with six replicates per plant species. Within generation medians for *L. bryoniae* life stages sharing the same letter did not differ significantly between host plants at P<0.05.

There were no significant differences in the size of the bean reared population across the three generations regardless of the life stage examined; however, for the tomato reared population there were significant differences both in the number of mines formed (χ^2 = 6.291, d.f. = 2, P=0.043), with generation three being less numerous than generation one (U=0.000, Z=-1.993, P=0.026) or two (U=0.000, Z=-1.993, P=0.046), and the number surviving to pupation (χ^2 = 7.218, d.f. = 2, P=0.027), with generation three being less numerous than generation one (U=0.000, Z=-2.334, P=0.026) or two (U=0.000, Z=-1.993, P=0.046), although there was no significant difference in the number of adults emerging. However, these differences become insignificant if only those lines which remained extant until the third generation are considered.

4.4.1.2. Liriomyza bryoniae size

The pupae used in generation zero as well as those formed in generations one, two and three were measured; a Kruskal-Wallis test showed that there were significant differences between generations in the size of pupae reared on tomato (χ^2 = 36.307, d.f. = 3, P<0.001) and bean (χ^2 = 39.084, d.f. = 3, P<0.001); from Figure 4-2 it is apparent that the pupae of tomato-reared *L. bryoniae* decreased in size over successive generations whilst the pupae of the bean-reared *L. bryoniae* increased in size. Comparisons of pupal size within generations and across host plants show that there was no difference in pupal size in generation zero ($F_{(1,118)}$=0.002, P=0.964) but that significant difference existed in generation one ($F_{(1,365)}$= 13.408, P<0.001), two ($F_{(1,467)}$= 52.899, P<0.001) and three ($F_{(1,304)}$=5.466, P=0.020).

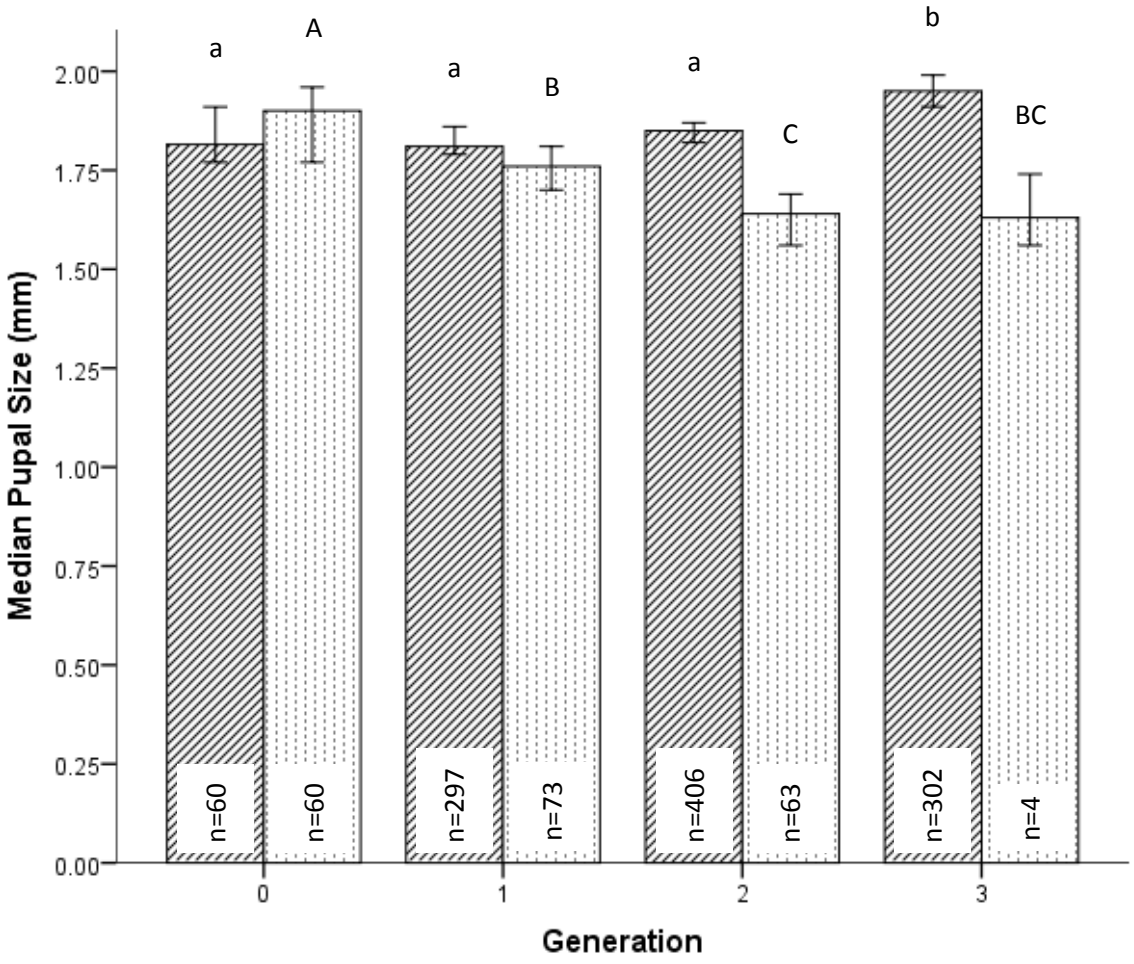

Figure 4-2: Median with 95% confidence intervals of the size (mm) of *L. bryoniae* pupae reared on the conventional culturing host; bean (diagonal striped), and on the novel host plant; tomato (dotted), over three generations. Within host species significant differences, at $P<0.05$, between generations are indicated by the letters at the top of each column, for bean (lower case) and tomato (upper case).

4.4.2. Effects of *L. bryoniae* host habituation on bean reared *D. isaea*

4.4.2.1. L. bryoniae

The parental generation of *L. bryoniae* was derived from 30 pupae placed into each cage, from which a mean number of 22.2 individuals emerged and this did not vary significantly between the host combinations ($F_{(2,6)}$=0.350, P=0.713). There were two potential ways of determining the population size of the offspring generation: the number of mines and the number of pupae. Comparisons revealed that there were significant differences between the host combination groups in the number of mines ($F_{(2,6)}$= 13.614, P=0.006), with 'Tomato *Liriomyza* in Tomato' having formed more mines than 'Bean *Liriomyza* in Bean' (P=0.006) or 'Bean *Liriomyza* in Tomato' (P=0.003) (Figure 4-3). Analysis of the numbers of larvae which pupated in each cage, however, revealed that the number of *L. bryoniae* available to *D. isaea* did not differ significantly between the host combinations ($F_{(2,6)}$ = 0.508, P = 0.626). Although the number of *L. bryoniae* consistently decreased between the larval (mine) and pupal stages, the decrease was only significant for the 'Tomato *Liriomyza* in Tomato' group (T=10.417, d.f.=2, P=0.009).

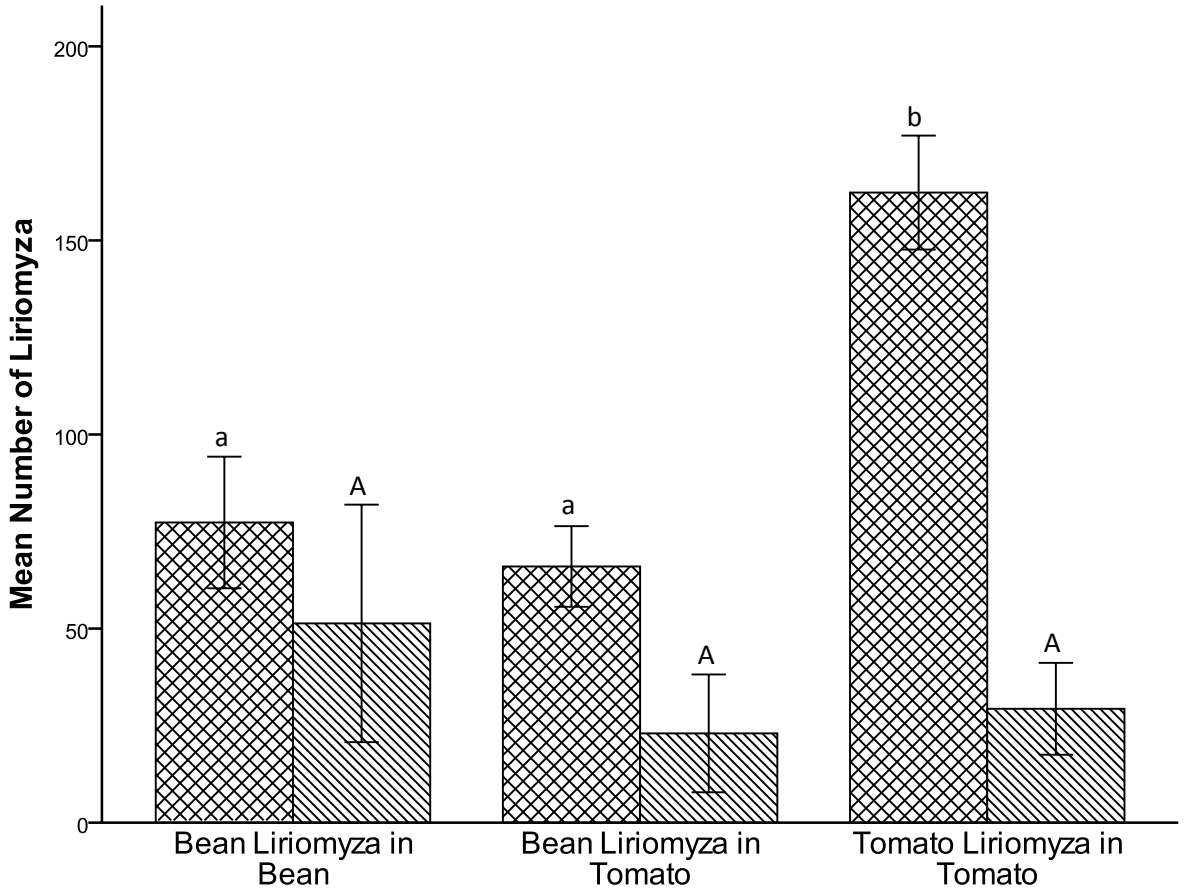

Figure 4-3: Mean ± standard error of the number of *L. bryoniae* mines (hatched) and pupae (diagonal stripe) per cage observed in each of the three host plant-*L. bryoniae* combinations, with three replicates per combination. Combinations sharing the same means, indicated by the letter above each bar, for mines (lower case) and pupae (upper case), are not significantly different at $P<0.05$.

Within the parental generation of *L. bryoniae* there were significant differences in the median size of pupae (χ^2 = 6.842, d.f. = 2, P=0.033) between host plant-*L. bryoniae* combinations, with the 'Bean *Liriomyza* in Tomato' pupae being significantly larger than the tomato-reared pupae (U=3158.00, Z = -2.552, P=0.011); there were no significant differences between other host plant-*L. bryoniae* combinations (Figure 4-4). In the offspring generation

there were also significant differences between host plant-*L. bryoniae* combinations ($F_{2,259}$=18.971, P<0.001), with the pupae of *L. bryoniae* from bean plants being significantly larger than those from tomato (P<0.001), whilst comparison revealed no difference between the two groups reared on tomato, despite the difference in their hereditary origin. Within the host plant-*L. bryoniae* combinations comparison between generations revealed that there were significant differences in the size of the parents compared to their offspring in the 'Bean *Liriomyza* in Bean' combination ($F_{1,242}$=41.742, P<0.001) and in the 'Tomato *Liriomyza* in Tomato' combination ($F_{1,176}$=7.387, P=0.007), but not between the generations of the 'Bean *Liriomyza* in Tomato' combination (Figure 4-4).

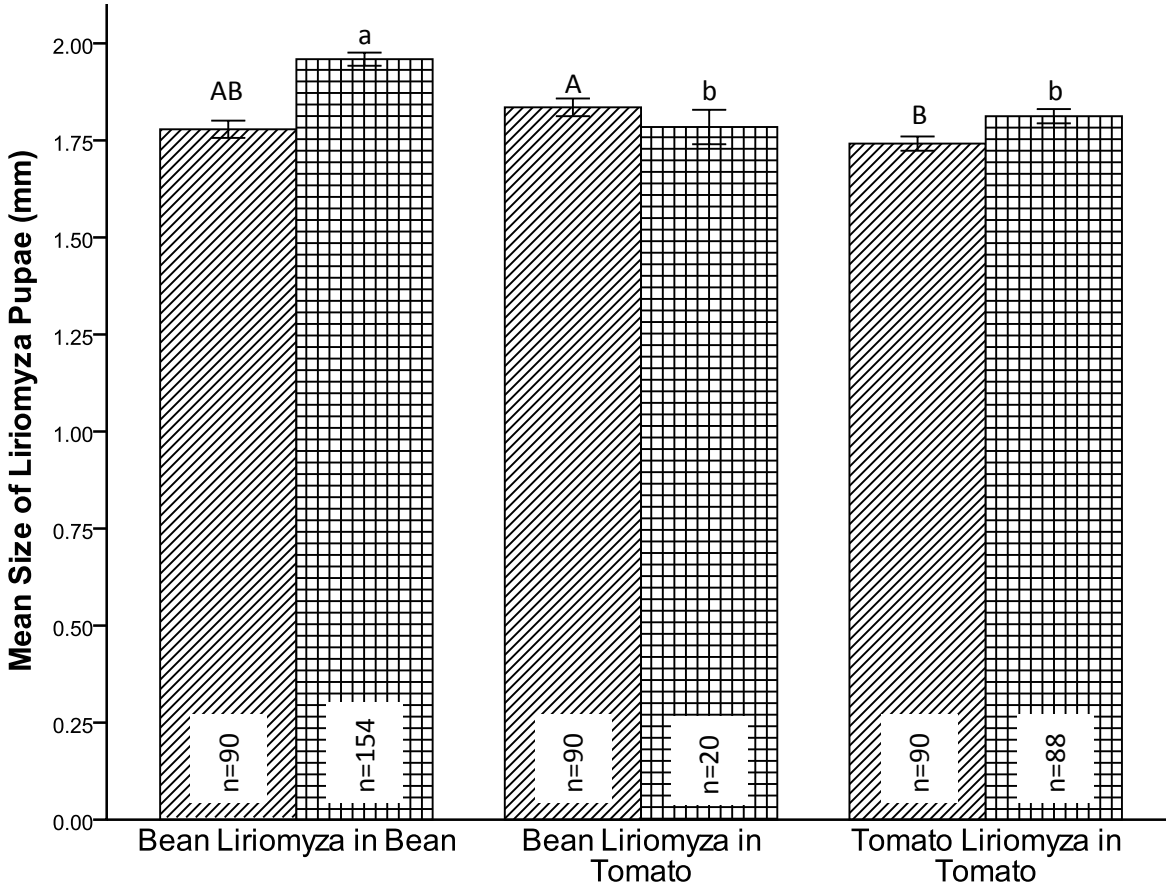

Figure 4-4: Mean ± standard error of the size of *L. bryoniae* pupae from two generations (diagonal stripe = parental generation, check = offspring) of the three host plant-*L. bryoniae* combinations. Upper case letters indicate groups of *L. bryoniae* in the parental generation which shared the same median size (χ^2 = 6.842, d.f. = 2, P=0.033), whilst lower case letters indicate groups of pupae in the offspring generation which shared the same mean size ($F_{2,259}$=18.971, P<0.001).

4.4.2.2. D. isaea

In total from across all replicates of the different host plant-*L. bryoniae* combinations 213 *D. isaea* emerged. Comparisons across the different host plant-*L. bryoniae* combinations reveal that there was no significant effect on the size of *D. isaea* populations ($F_{2,6}$ =2.583, P = 0.155), (Figure 4-5).

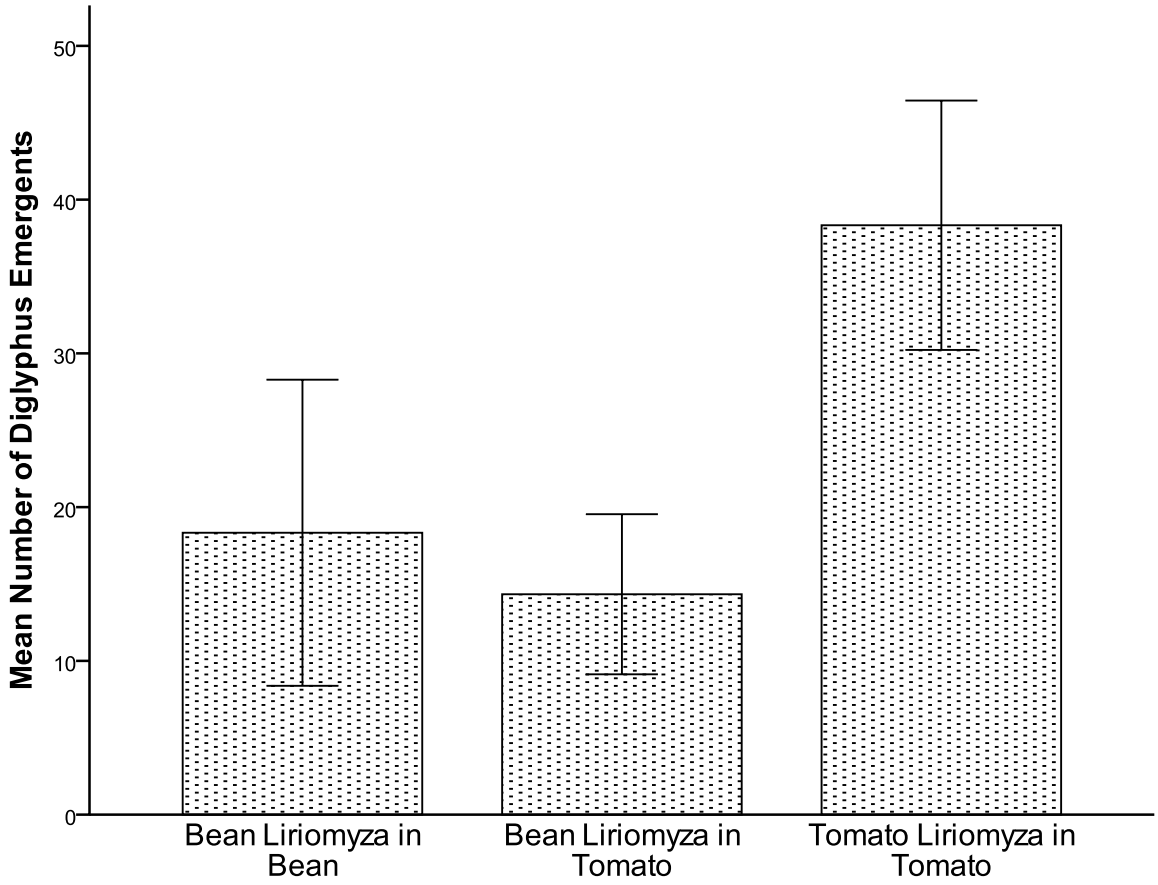

Figure 4-5: Mean ± standard error of the number of *D. isaea* adults emerging from the three host plant-*L. bryoniae* combinations, with three replicates per combination. At P<0.05 the number of *D. isaea* did not differ significantly between combinations.

Male *D. isaea* were significantly smaller than females (U=2559.5, Z=-4.705, P<0.001) for this reason analysis of *D. isaea* size was been conducted separately for males and females. Comparisons reveal that there was no significant effect of host combination on either male ($F_{2,110}$=2.004, P=0.140) or female size ($F_{2,73}$=1.000, P=0.272) (Figure 4-6).

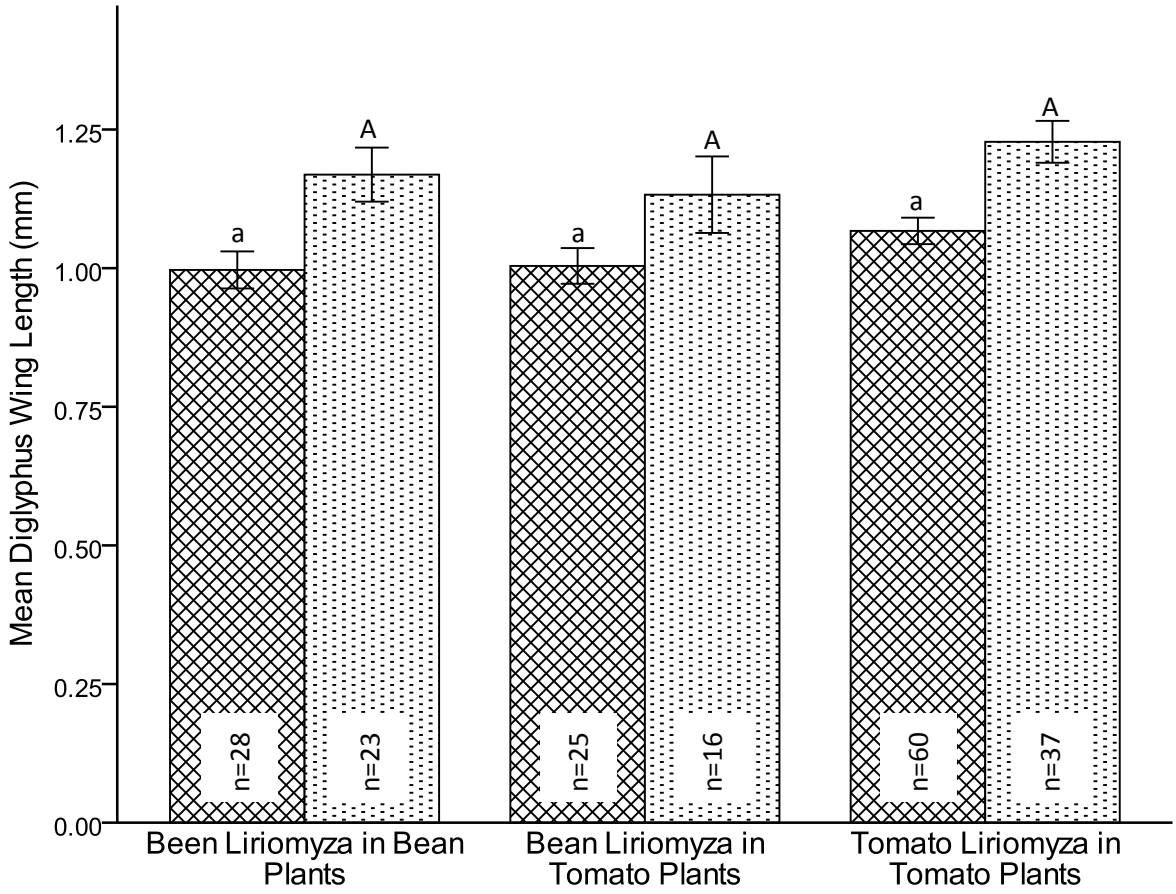

Figure 4-6: Mean ± standard error of the wing length of *D. isaea* males (hatched) and females (doted) emerging in cages of the three host plant-*L. bryoniae* combinations. Combinations sharing the same means, indicated by the letter above each bar, for males (lower case) and females (upper case), are not significantly different at $P<0.05$.

Log-likelihood goodness-of-fit tests have been used to analyse the hypothesis that the observed sex ratios in each class differed from the expected 1:1 ratio, with Williams' corrections applied to classes which had less than two hundred cases (Sokal & Rohlf 1981, Heimpel & Lundgren 2000). There was no significant deviation from the expected proportions of males to females in any of the groups (Figure 4-7) when significance levels

were determined using χ^2 distributions at one degree of freedom: G=2.19, 1.13, 1.47 for *D. isaea* sex ratios on 'Bean *Liriomyza* in Bean', 'Bean *Liriomyza* in Tomato' and 'Tomato *Liriomyza* in Tomato' respectively.

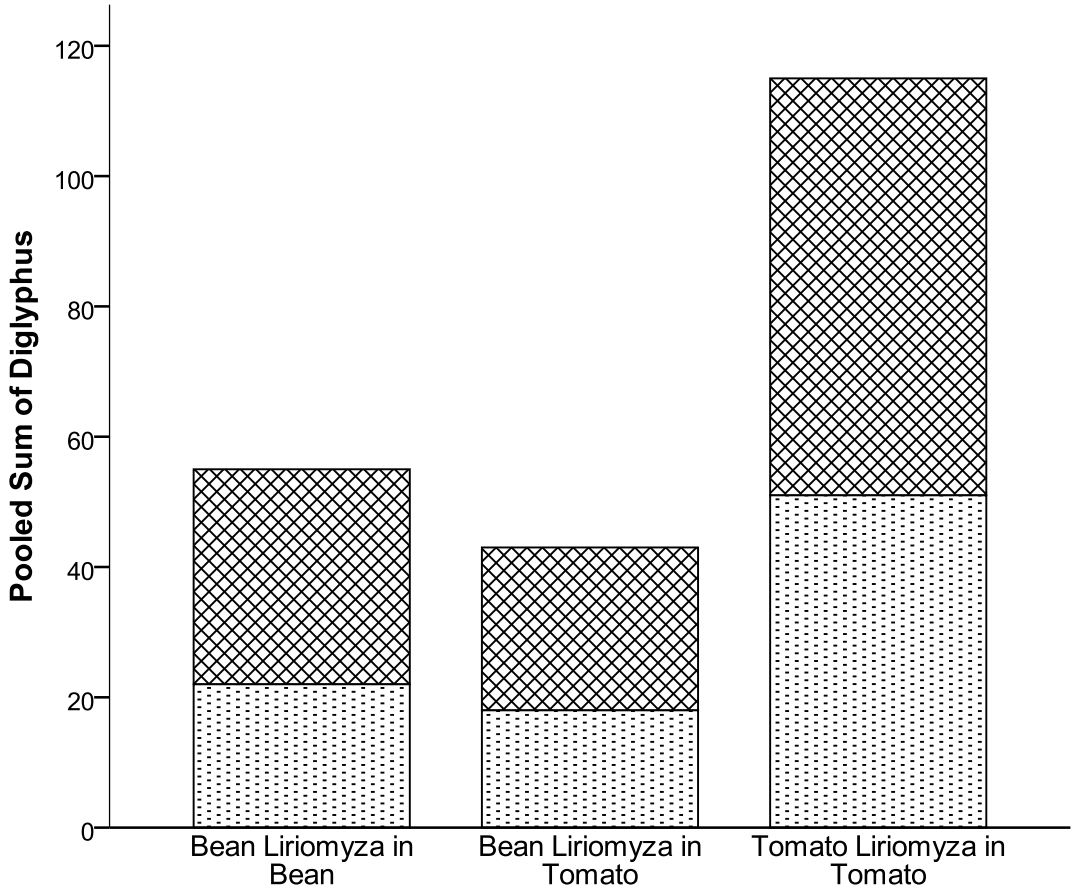

Figure 4-7: Pooled sum of male (hatched) and female (dotted) *D. isaea* according to the host combination on which the populations were reared. At P<0.05 there was no significant deviation from a proportion of 0.5 males in any of the host combinations.

4.4.3. Effects of natal host on *D. isaea*

4.4.3.1. L. bryoniae

The origin of the parental *D. isaea* had no significant effect on the number of *L. bryoniae* larvae in a cage (Figure 4-8); however, *D. isaea* origin did have a significant effect on the number of *L. bryoniae* larvae which survived to pupation ($F_{(1.4)}$=12.096, P=0.025).

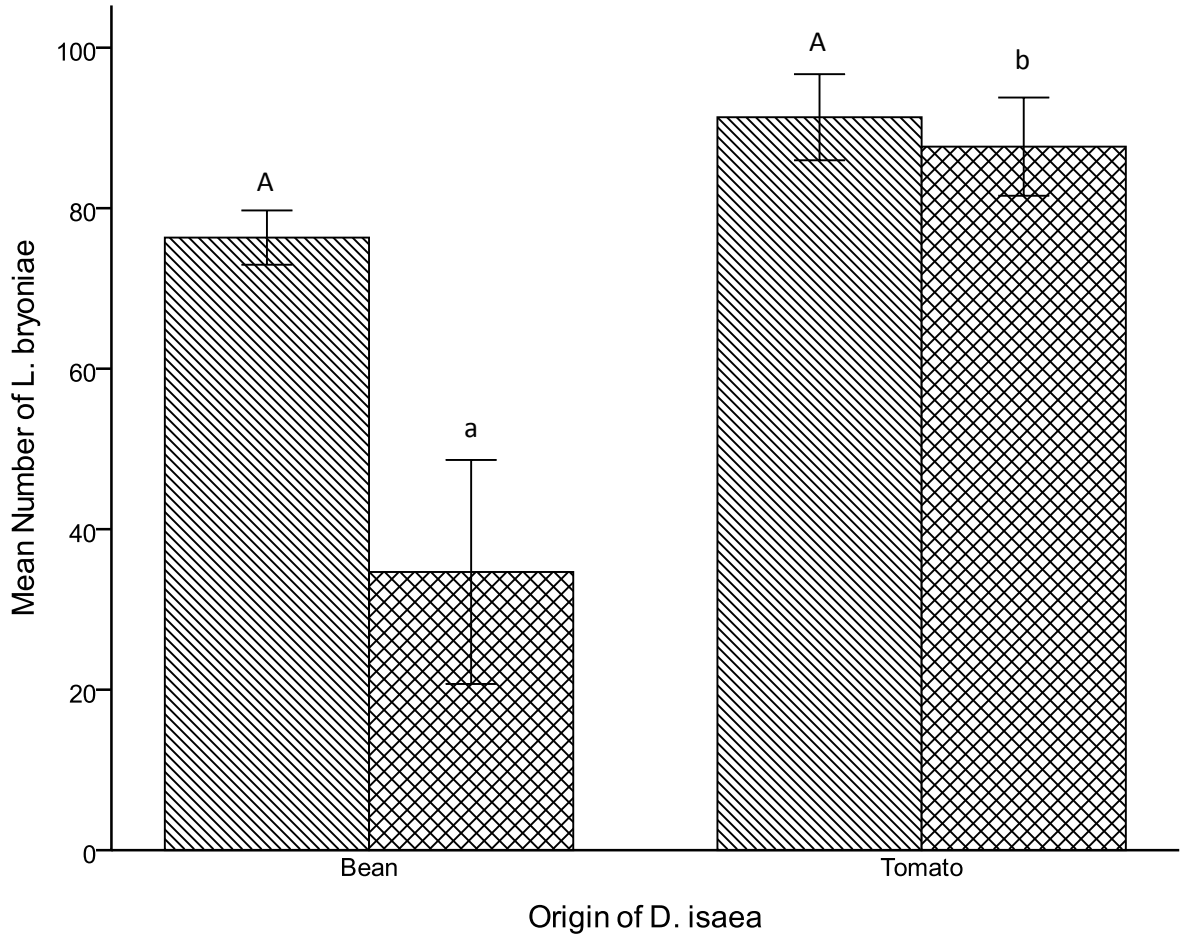

Figure 4-8: Mean ± standard error of the number of *L. bryoniae* larvae (diagonal stripe) and pupae (checked) counted in tomato with *D. isaea* from either a conventional bean culture or reared on tomato for one generation, with three replicates per treatment. Means sharing the same letter (uppercase for larvae & lowercase for pupae) were not significantly different at P<0.05.

4.4.3.2. D. isaea

The number of *D. isaea* in the offspring generation was unaffected by the origin of the parental *D. isaea*, both for the total number of offspring per replicate and for the separate genders. Log-likelihood goodness-of-fit tests have been used to analyse the hypothesis that the observed sex ratios in each class differed from the expected 1:1 ratio, with Williams' corrections applied to classes which had less than two hundred cases (Sokal & Rohlf 1981, Heimpel & Lundgren 2000). There was no significant deviation from the expected proportions of males to females in the offspring of the bean-reared *D. isaea*; however when significance levels were determined using χ^2 distributions at one degree of freedom a significant variation from the expected sex ratio was found in the offspring of the tomato-reared *D. isaea* (G=5.105, P=0.024) with males being more numerous than females (Figure 4-9).

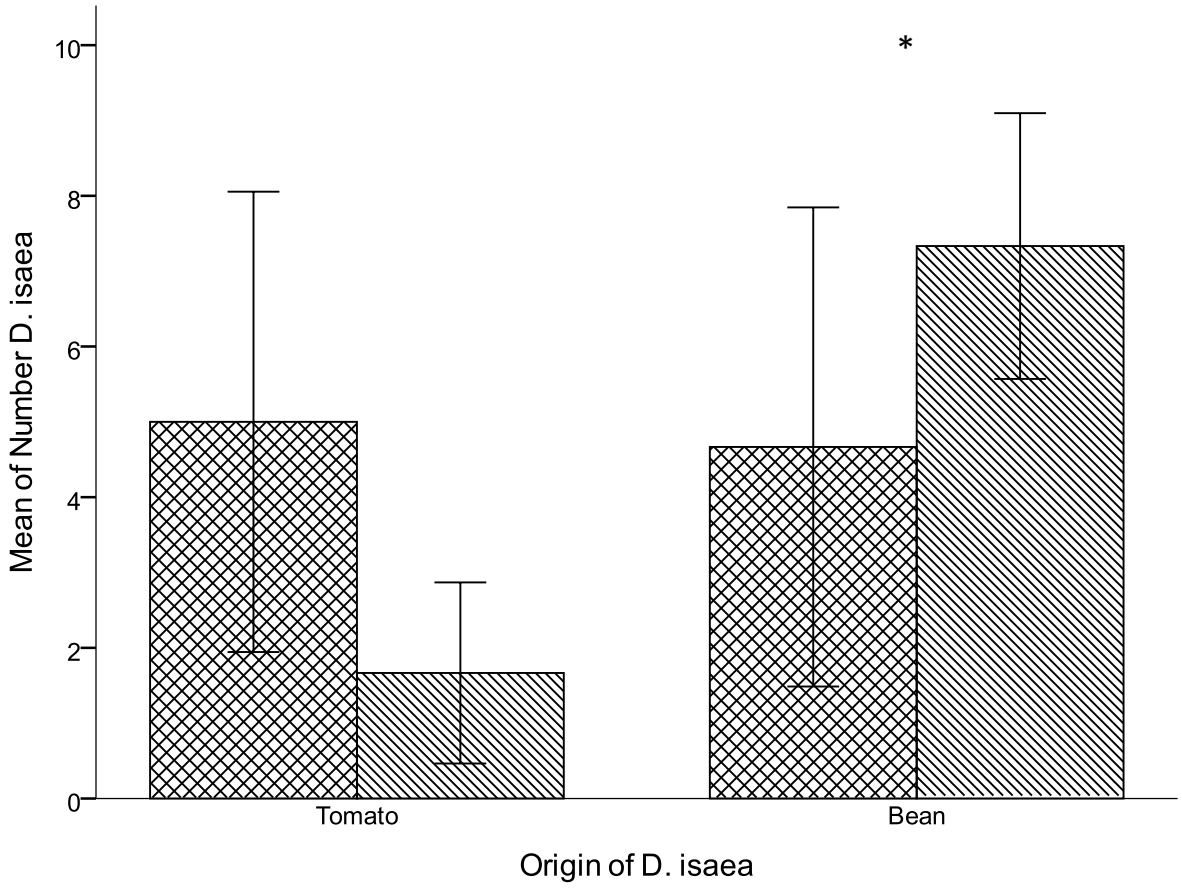

Figure 4-9: Mean ± standard error of the number of *D. isaea* offspring per cage according to the natal plant origin of the parental *D. isaea,* males (hatched) and females (diagonal stripe). Stared columns indicate a significant development from the expected proportion of 0.5 males at $P<0.05$.

A three-way ANOVA indicated that the gender of *D. isaea* has a significant effect on wing length ($F_{(1,88)} = 22.891$, $P<0.001$); however, neither generation, the natal origin of *D. isaea* nor any of the interaction terms are significant (Figure 4-10).

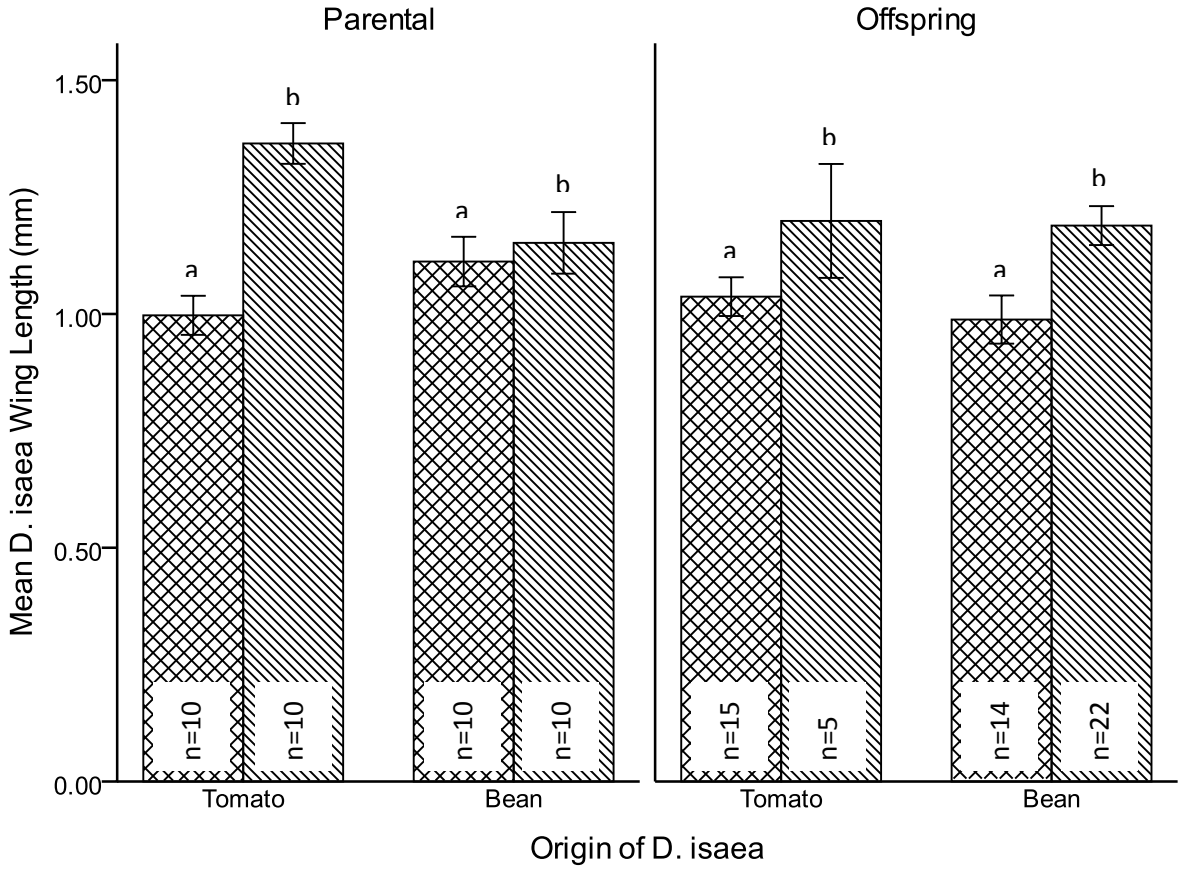

Figure 4-10: Mean ± standard error of the wing length (mm) of adult *D. isaea* from both the parental and offspring generations according to the natal plant origin of the parental *D. isaea*; males (hatched) and females (diagonal stripe). Means with the same letter indicate genders which did not differ significantly at $P<0.05$, wing length did not differ significantly according to generations or natal origin of parent.

4.5. Discussion

At the species level *L. bryoniae* is well known for being highly polyphagous, feeding on a wide range of vegetable and ornamental crop plants across a number of plant orders

(Minkenberg & van Lenteren, 1986). Polyphagy has been defined as 'indiscriminate feeding on a number of plant species belonging to different plant orders' (Spencer 1964 cited in: Minkenberg & van Lenteren, 1986) and is synonymous with generalism. At the opposite end of the spectrum are specialised species which feed on only a single or small group of closely related species (Roslin & Salminen, 2008). It is generally assumed that specialists are more efficient than generalists as the latter have to maintain metabolic machinery capable of tolerating, detoxifying or metabolising a broader spectrum of potentially noxious chemical compounds than will be encountered by specialists, and it is assumed that there must be a physiological cost, in terms of reduced efficiency, for maintaining this metabolic machinery (Fox & Morrow, 1981). It could therefore be assumed that after 14 generations of being cultured solely on bean at in Birmingham laboratories, and possibly for many decades previously in culture at Koppert B.V. (The Netherlands), it would no longer be adaptive for this population of *L. bryoniae* to maintain its polyphagous capability. However this is not the case and as can be seen from Figure 4-1 *L. bryoniae* was still capable of recognising tomato as a potential host plant and a new generation of viable *L. bryoniae* were able to develop in those plants. Similarly Martin *et al.* (2005) found that after 8-12 generations of rearing *L. huidobrensis* solely on a single host plant there was no impact on the preference of those insects for their culturing host over other potential host plants.

The transfer of long term bean-cultured *L. bryoniae* to tomato is not without cost; the tomato reared populations of *L. bryoniae* have greater larval mortality, and as a result produce fewer pupae and adults (Figure 4-1), and show a greater tendency to go extinct, with three of the tomato populations dying out between the first and second generations compared with just one bean population. There are also costs at the level of the individual

insect with *L. bryoniae* reared from bean consistently attaining a greater pupal size than those reared from tomato, across generations one to three (Figure 4-2). Similarly the tephritid fruit fly, *Rhagoletis pomonella*, showed morphological changes when incorporating a novel plant (apple), into its range of hosts, with changes in body size, number of postorbital bristles and ovipositor length observed. Due to changes in phenology associated with the shift of some *R. pomonella* to apple, offspring from this host have rapidly become isolated from their siblings on the original host of hawthorn (Bush, 1969). However, due to its mulitvoltine nature and the overlapping of generations this level of isolation on different host plants is unlikely to occur rapidly in *L. bryoniae*.

Intriguingly, the pupal size of *L. bryoniae* reared in tomato decreases consistently across generations until it stabilises between the second and third tomato-reared generation, suggesting a possible adaptive advantage of small size in the tomato-reared population. Tavormina (1982) also found evidence indicative of host related divergence in populations of *L. brassicae* reared on different plants, however, as in the current study, these differences between populations might have been accentuated by forced positive mating preventing the interbreeding between flies reared on different hosts which would be expected to occur in the wild.

The concern for biological control practitioners is not how pests, such as *L. bryoniae*, adapt to different host plants, but whether there is any implication for species in the third tropic level which may be used as biological control agents. The second experiment in this series therefore focused on that issue, using three combinations of *L. bryoniae* and host plant: 'Bean *Liriomyza* in Bean', a population of *L. bryoniae* which for many generations had been

reared on bean and in this experiment were offered bean as a host; 'Bean *Liriomyza* in Tomato', a group from the same population which in this experiment were offered tomato as a host plant; and, 'Tomato *Liriomyza* in Tomato', a population derived from the original bean population but which had been reared on tomato plants for four generations and in this experiment were offered tomato as a host plant. *Diglyphus isaea* from the standard bean reared stock culture were offered these three host combinations in a non-choice experiment and comparisons of realised fecundity, individual size and sex ratio were made between the three host combinations.

The three populations of *L. bryoniae* showed significant differences in abundance at the larval stage with 'Tomato *Liriomyza* in Tomato' producing a greater number of mines that either of the bean derived populations. However, at the pupal stage there was no significant difference in population size between the three groups (Figure 4-3). Of the three *L. bryoniae* populations, only 'Tomato *Liriomyza* in Tomato' showed a significant decrease in population size between the larval and pupal stages. There are two possible explanations for this: firstly, *D. isaea* may have used a greater proportion of the *L. bryoniae* larvae from this host combination for host feeding and parasitation, or secondly; despite four generations of habituation to tomato, *L. bryoniae* may still suffer from increased larval mortality on this host. As mines were not dissected in this experiment it is impossible to be sure of *D. isaea* host feeding patterns, but with reference to the previous experiment the was high larval mortality in tomato-reared *L. bryoniae* into the third generation with no evidence of that trend subsiding (Figure 4-1).

Diglyphus isaea population size did not differ significantly between the three host combinations. Although *D. isaea* populations were greatest in the 'Tomato *Liriomyza* in Tomato' group (Figure 4-5) there was no evidence that *D. isaea* was responsible for the increased *L. bryoniae* larval mortality in that group. These results do however suggest that *D. isaea* is equally effective as a control agent of *L. bryoniae* regardless of whether the host plant is bean or tomato and the number of generations *L. bryoniae* has spent habituating to that host plant. Conversely, the fecundity of the predatory mirid *Macrolophus caliginosus* was found to be significantly affected by the host plant on which the prey *Aleyrodes proletella* was reared, with a significantly greater number of eggs being laid on Chinese cabbage than on cabbage or Brussel sprout (Hatherly *et al.*, 2009).

Bottrell *et al.*, (1998) suggest that reduced performance by a pest species need not diminish the efficacy of a biological control agent unless the reduction in population growth potential of the predator or parasitoid is greater than that of the pest. This appears to be the case in the current study, where by host plant affects insect size, a correlate of fitness (Honek, 1993), in the pest, *L. bryoniae* (Figure 4-4), but not in the parasitoid, *D. isaea* (Figure 4-6). This suggests that despite the host related size differences in *L. bryoniae*, the larvae from all three populations provided developing *D. isaea* with sufficient resources to achieve their maximum potential size. Similarly, Starks *et al.* (1972) showed that although there were fewer greenbugs, *Schizaphis graninum*, and they were smaller on resistant strains of barely than on susceptible strains, the presence of the parasitoid *Lysiphlebus testaceipes* was still beneficial for greenbug control.

Hatherly *et al.*, (2009) suggest that in order for host acceptance to have occurred, a predator, or as in the current study a parasitoid, must survive to adulthood and successfully reproduce. As *D. isaea* has successfully been reared from *L. bryoniae* in bean for many generations in the laboratory stock culture at The University of Birmingham, it can be asserted that bean-reared *L. bryoniae* is an accepted host for this population of *D. isaea*. However, as glasshouse tomatoes are the target crop which *D. isaea* is expected to protect, the final experiment in this series examined whether *D. isaea* could successful survive in tomato into the second generation and what affect natal host, bean or tomato, may have on population size, insect body size and the sex ratio of *D. isaea*.

The most important observations, in terms of *D. isaea*'s efficacy as a biological control agent, from this final study are that *D. isaea* reared from tomatoes are capable of producing offspring in equal numbers (Figure 4-9) and of an equal size (Figure 4-10) to *D. isaea* reared on bean, with tomato as a host plant. However, despite all other causes of variance (the host plant, the origin of *L. bryoniae* and the number of mines) being equal, the reduction in *L. bryoniae* surviving to pupation in replicates treated with bean-reared *D. isaea* suggests that the bean-reared *D. isaea* used more larval *L. bryoniae* for parasitisation and host feeding than did the tomato-reared *D. isaea*. This is not, however, consistent with the observation that there was no significant difference in the number of *D. isaea* which emerged in the offspring generation from the two treatments unless the offspring of the bean-reared *D. isaea* experienced higher mortality that the offspring of the tomato-reared *D. isaea*. In this experiment the *L. bryoniae* mines were not dissected so it is not possible to ascertain the relative importance of host feeding versus parasitism by *D. isaea* in contributing to the increased *L. bryoniae* mortality in the cages containing bean-reared *D.*

isaea; higher mortality among bean-reared *D. isaea* may be caused by the presence of potentially toxic alleochemicals in tomato plants (Campbell & Duffey, 1979) exerting a selective pressure through which the tomato-reared *D. isaea* had passed in the preceding generation. Indeed, Sznajder and Harvey (2003) suggested that toxicity in host plants causes natural enemy species to become 'partitioned' into populations which are or are not adapted to exploit prey/hosts on those host plants. If, however, the increased mortality of *L. bryoniae* in the cages of bean-reared *D. isaea* was caused by host feeding it is unapparent whether this would be positive or negative from a biological control perspective (Kidd & Jervis, 1989).

The sex ratio in the offspring generation of the *D. isaea* reared from tomato unlike that of the *D. isaea* reared from bean varies from the anticipated ratio of 1:1 and is male biased. Similarly, Morris and Fellowes (2002) also found that the parasitoid *Pachycrepoideus vindemiae* had a male biased sex ratio when reared on its natal host and attributed this to the fact that parasitoids have the capacity to determine the gender of their offspring through the haplo-diploid determination of sex (Flanders, 1956) and that female eggs are deposited on higher quality hosts. Host quality is known to be assessed on a relative basis (Charnov *et al.*, 1981); Morris and Fellowes (2002) suggest that natal host, through an interaction with parental size, contributes to how an ovipositing female assesses host quality. It is likely that a similar mechanism has contributed to the discrepancy in the sex ratios of *D. isaea* displayed in the current study.

In conclusion, long term rearing exclusively on bean has not affected the ability of *L. bryoniae* to colonise the alternative host plant of tomato; however, even after three

generations of habituation to this new host, *L. bryoniae* still experience increased larval mortality compared with populations on the ancestral host of bean, and those *L. bryoniae* which do succeed in pupating from tomato are consistently smaller. The habituation of *L. bryoniae* to tomato does not appear to affect *D. isaea* with both populations and individuals attaining equal size on bean-habituated *L. bryoniae*, tomato habituated *L. bryoniae*, and *L. bryoniae* in the first generation transferred to new the host of tomato. However, unlike *L. bryoniae*, *D. isaea* did not show significant negative effects of rearing on tomato with fitness correlates including number of offspring and size of offspring being equal to *D. isaea* reared on bean. From a biological control perspective this indicates that the mass rearing of *D. isaea* on bean for use as a control agent in commercial tomato crops should not be a cause for concern.

5. *SOLANUM LYCOPERSICON* ONTOGENETIC STAGE AFFECTS HERBIVOURY WITH CONSEQUENCES FOR THE THIRD TROPIC LEVEL.

5.1. Abstract

Plants pass through four ontogenetic stages: seedling, juvenile, mature and senescent. Between stages there are quantitative and qualitative changes in both the anti-herbivore defences and nutritional value of a plant; the nature of these changes however varies across the plant kingdom. Further to which, indirect plant defences such as the production of natural enemy attracting volatiles also change with ontogenetic stage. This study examines how the ontogenetic stage of tomato, *Solanum lycopersicon*, affects both the herbivorous leaf mining pest *Liriomyza bryoniae* and its parasitoid *Diglyphus isaea*. *Liriomyza bryoniae* forms more leaf punctures and mines in juvenile than mature plants, however, *L. bryoniae* developing on mature plants attain greater size. The presence or absence of *D. isaea* had no effect on *L. bryoniae* larvae, pupae or adult numbers. *Diglyphus isaea*, however, developed only on *L. bryoniae* in juvenile plants. Possible causes for the differences in *L. bryoniae* and *D. isaea* between juvenile and mature tomato plants are discussed, as well as the implications for the biological control of *L. bryoniae* by *D. isaea* in this crop.

5.2. Introduction

As plants grow they pass through four distinctive ontogenetic stages: seedling, vegetative juvenile, mature and senescent. Although the boundaries between progressive stages are indistinct, the four stages are recognisable from definable characteristics; seedlings are dependent on reserves stored within their seed, the juvenile stage begins once seed reserves are exhausted, maturity occurs as preparation for reproduction is initiated e.g. flower bud formation, whilst senescence is the terminal stage of degradation leading to death (Barton & Koricheva, 2010; Nooden *et al.*, 1997). There are changes in both levels and types of anti-herbivore defences used between ontogenetic stages; however, there is no universal pattern across the plant kingdom in the level or type of defence used in each stage (Barton & Koricheva, 2010).

There are also changes in the levels of plant nutrients available to herbivores dependent on the ontogenetic stage of the plant on which they are feeding; for instance, in the shrub *Erythoxylum tortuosum*, younger leaves were found to have higher nitrogen and water contents than older leaves (Ishino *et al.*, 2011). However, as with defensive compounds and structures this pattern may not be uniform across the whole of the plant kingdom, as the converse pattern is seen in clones of aspen, *Populus tremuloides*, where leaf nitrogen content increased with maturity (Donaldson *et al.*, 2006). These changes in the chemical constituents of plants through their ontogeny are accompanied by changes in plant size, architectural complexity and root: shoot ratio (Boege & Marquis, 2006), meaning that as the nutritional quality of the plant decreases, the quantity of the plant resource increases, creating a trade-off for herbivores.

Besides the 'bottom-up' regulation that plant quality and quantity impose on herbivore populations there is also 'top-down' regulation of these population exerted by predation (Boege & Marquis, 2005). Plants are responsible for influencing 'top-down' regulation through traits such as size, structural complexity and signalling to natural enemies. In order to attract arthropod predators and parasitoids, plants damaged by herbivores release a cocktail of volatile chemicals, known as Herbivore-Induced Plant Volatiles (HIPVs), Arthropod natural enemies are able to detect these HIPVs and use them as long range cues to identify plants that may be infested with potential prey/hosts (Hare, 2011). HIPVs can vary not only according to the plant species under attack and the species of herbivore (Hare, 2011) but also according to the ontogentic stage of the plant; for instance, juvenile soybeans, *Glycine max*, produced 10-fold more volatiles than mature reproductive plants when under attack by caterpillars of the lepidopteran species, *Spodoptera frugiperda*, (Rostás & Eggert, 2007).

The preceding chapters have concentrated on how the host plant species can impact on the ability of *L. bryoniae* to utilise that plant as a host and in turn how this affects the efficacy of *D. isaea* as a control agent of *L. bryoniae*. As detailed above, it is not only the species of plant that can effect herbivores and their predators or parasitoids but also the ontogentic stage of the plant, however, due to the inconsistency in patterns of defence by plants and the nutritional value of those plants to insect herbivores between the ontogentic stages of plant development, it is unclear how individuals and populations of the leaf mining fly *Liriomyza bryoniae* may be affected by the ontogentic stage of their host plant tomato, *Solanum lycopersicon*. Nor is it apparent what impact this may have on the ability of the parasitoid *Diglyphus isaea* to utilise *L. bryoniae* as a host? These questions are of specific importance because *L. bryoniae* is an economically important pest of tomatoes in European glasshouse

agriculture and because *D. isaea* is commonly used as a biological control agent of *L. bryoniae*. Although the efficacy of *D. isaea* is reported to vary seasonally (pers. com. J. Klapwijk), the work discussed in this chapter tested the hypothesis that it is the ontogenetic stage of the tomato plant and not seasonality itself that is responsible for variations in *D. isaea* efficacy.

5.3. Material and Methods

5.3.1. Insect culture

All insect material used in this series of experiment was derived from the laboratory populations maintained at the University of Birmingham, see Chapter 2: Insect Culture.

5.3.2. Plant material

Tomato, *Solanum lycopersicon* L. (also known as *Lycopersicon esculentum Mill.) var. Elanto rz*, plants were grown at Winterbourne Gardens, University of Birmingham. Throughout the experiment plants were maintained and watered *ad libitum*.

The group of older plants were sown 48 days prior to the start of the experiment and showed the characteristics of maturity at the onset of the experiment. The younger plants, were sown 28 days prior to the start of the experiment and these were in the juvenile stage at the start of the experiment.

5.3.3. Environmental Conditions

The experiment described in this chapter was performed in a glasshouse environment at Winterbourne Gardens. The temperature of the glasshouse was set at 20°C although it

varied during the course of the experiment from 7°C to 30°C. No supplementary lighting was required during the experiment.

5.3.4. Plant Size Experiment

In February 2012, six replicates of three mature plants (Photograph 5.1) were established in cages (33cmW x 58cmL x 113cmH) and six replicates of three juvenile plants (Photograph 5.1) were in smaller cages (60cmW x 60cmL x 60cmH). The cages were constructed from fiberglass poles covered in fine muslin and laid out alternately large then small along the length of the glasshouse. At the outset of the experiment 16 newly emerged (<48hrs) adult *L. bryoniae* (7 males and 9 females) were added to each cage and thereafter were allowed to mate, feed and oviposit freely for the whole of their natural lives. On the 10th day the number of *L. bryoniae* mines and feeding/oviposition punctures on the leaves were counted by eye. Paper collars were also fitted around the base of the plants so that the *L. bryoniae* larvae emerging from the leaves, in order to pupae, would be directed onto the base of the cage from where they could easily be collected. On the 11th day three female and one male newly emerged adult *D. isaea* were added to half of the cages, the remaining cages acted as controls. From the 14th day *L. bryoniae* pupae were collected; these were counted and stored on damp tissue until adult emergence. The first *L. bryoniae* adults emerged on the 23rd day; these were collected using an aspirator and then frozen before being counted, sex and photographed in order that the length of the wings could be measured using the software package ImageJ. On the 28th day the first newly emerged adult *D. isaea* were collected and as with the *L. bryoniae*, these were frozen before being counted, sex, photographed and measured. The final *D. isaea* adults emerged on the 39th day.

Photograph 5.1: Tomato plants, mature plant (left), approximately 70 cm tall (from soil surface to apical tip) and showing signs of preparation for reproduction e.g. flower bud formation. Juvenile tomato plant (right), approximately 10 cm tall (from soil surface to apical tip) with true leaves but not yet preparing for reproduction.

5.3.5. Statistical Analysis

Statistical analysis was performed using SPSS 20 (IMB). One and Two way ANOVAs were performed as appropriate. Where data failed to meet the assumptions of Two-way ANOVA a Scheirer-Ray-Hare test was employed. The sex ratios of emergent *D. isaea* was analysed using log-likelihood goodness of fit tests with William's correction (as described in section 3.3.8).

5.4. Results

5.4.1. Plants

At the start of the experiment the 48 day old mature tomato plants had a mean height of 69.1cm tall, 59.7cm taller than the juvenile plants, sown 24 day prior to the start of the experiment, which had a mean height of 9.4cm. After 39 days at the conclusion of the experiment the mature plants had attained a mean height of 122.2cm whereas the juvenile plants had grown only to 56.2cm tall (Figure 5-1); at the end of the experiment the younger plants also showed characteristics of maturity. A Scheirer-Ray-Hare test revealed that both plant group, mature or juvenile (d.f.=1, SS=71108.490, H=12.293, P<0.001), and time, initial or final (d.f.=1, SS=44965.007, H=7.773, P=0.005), had a significant effect on plant height, although the interaction between these factors was not significant.

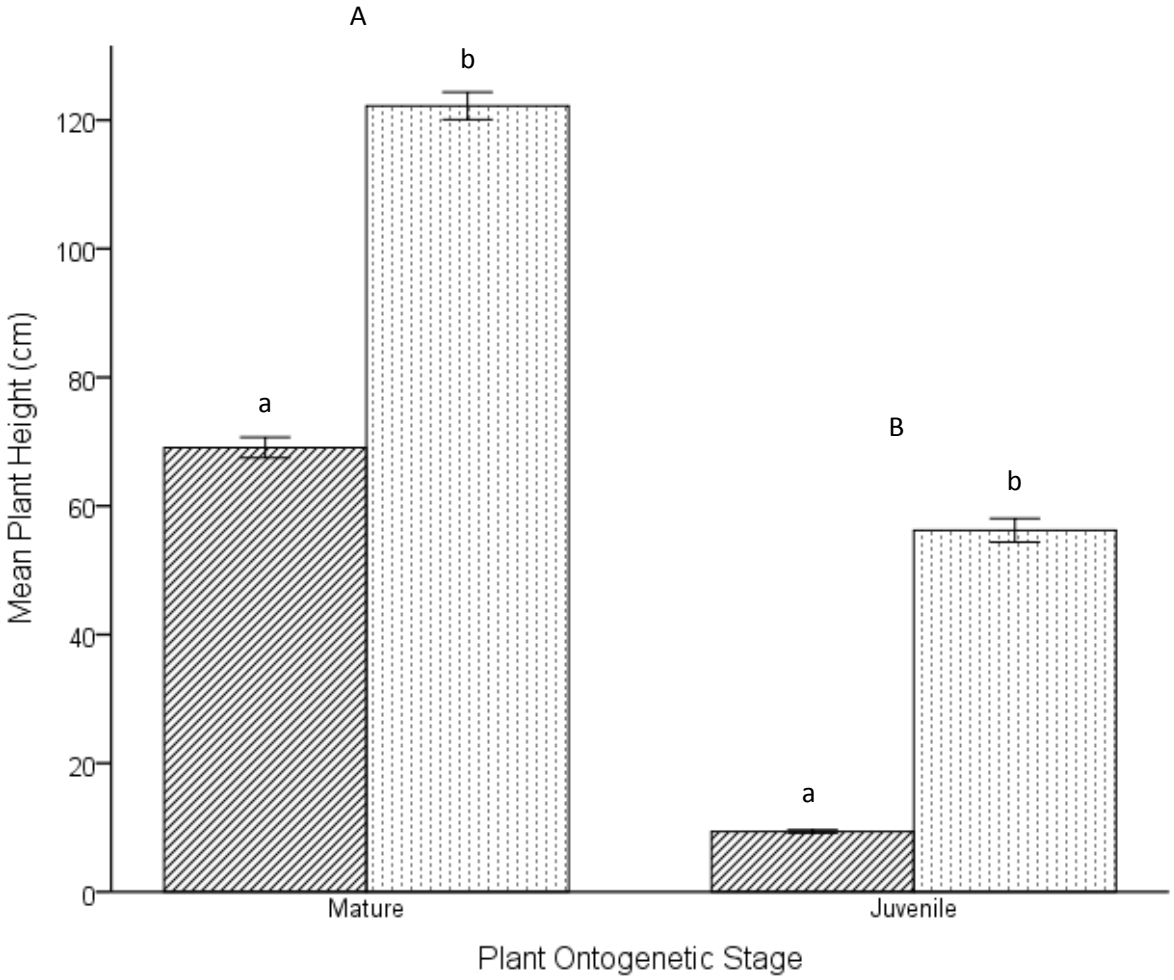

Figure 5-1: Mean ± standard error of the plant height (cm) for the two groups of plants, mature and juvenile, both at the outset (initial height, diagonal stripe) and at the end of the experiment (final height, dotted), with six replicates at each ontogenetic stage. Means with the same letter (uppercase for between ontogenetic stages and lowercase for initial and final heighs within ontogenetic stage) were not significantly different at $P<0.05$.

5.4.2. *Liriomyza bryoniae*

Leaf punctures formed by the adult females *L. bryoniae* of the parental generation for feeding and oviposition varied significantly according to the ontogenetic stage of the tomato

plants ($F_{(1,10)}$=47.612, P<0.001) with juvenile plants suffering the greater number of punctures (Figure 5-2).

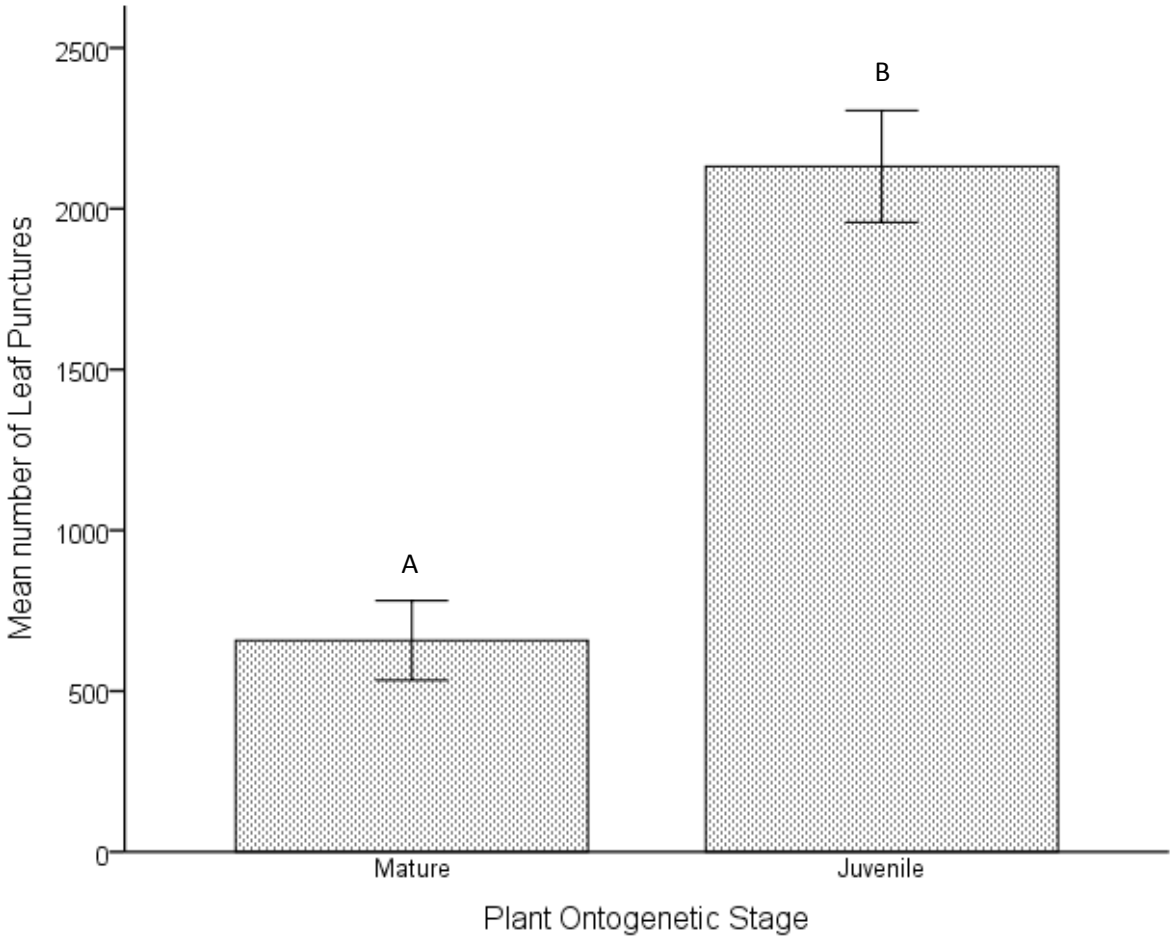

Figure 5-2: Mean ± standard error of the number of leaf punctures (feeding and oviposition) per cage formed by adult *L. bryoniae* in the parental generation on mature and juvenile plants, with six replicates per ontogenetic stage. Means with the same letter are not significantly different at P<0.05.

There was also significant variation in the numbers of *L. bryoniae* mines ($F_{(1,10)}$=83.890, P<0.001), pupae ($U_{(12)}$=0.000, Z=-2.898, P=0.004) and emergent adults ($F_{(1,10)}$=16.614,

P=0.002) according to the ontogenetic stage of the host plant, again with cages of the juvenile plants always containing the greater number (Figure 5-3).

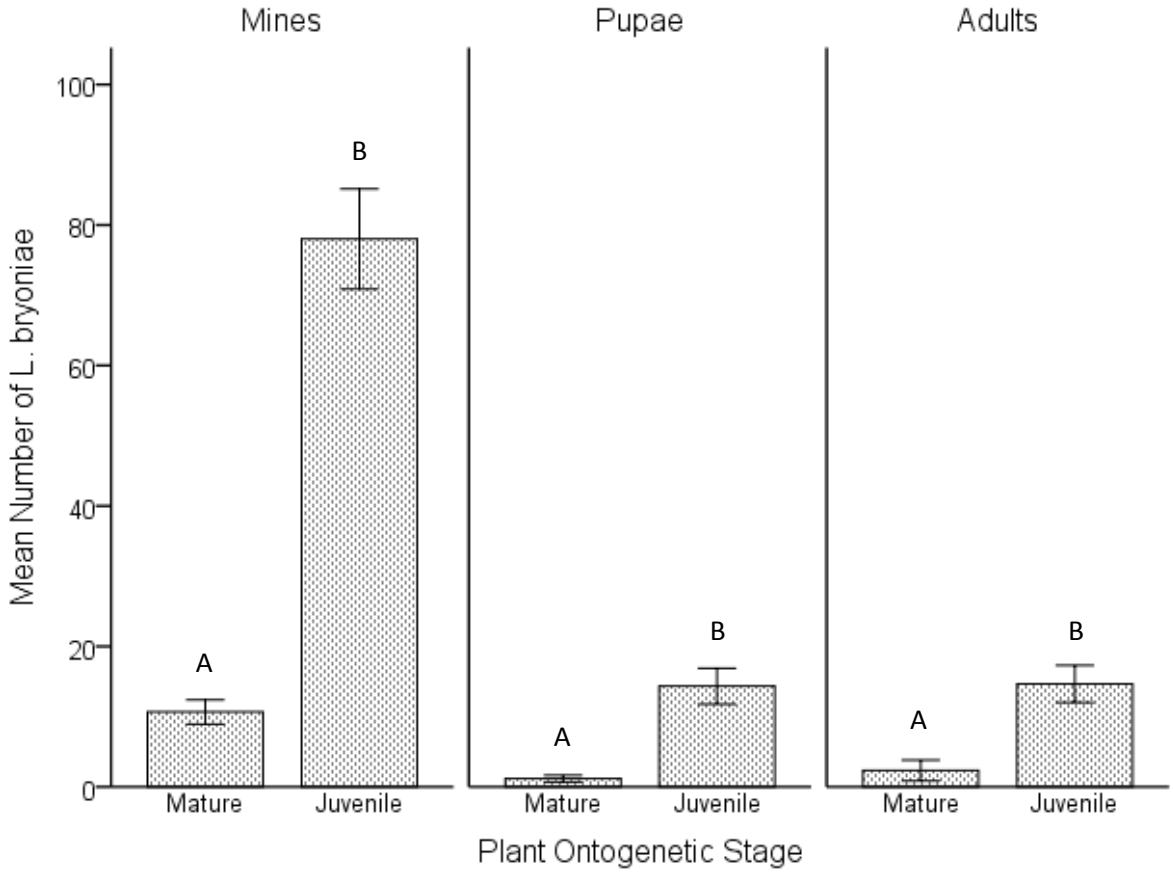

Figure 5-3: Mean ± standard error of the number of *L. bryoniae* in each life stage (mine, pupa and adult), originating from cages of mature and juvenile plants, with six replicates per ontogenetic stage. Within *L. bryoniae* life stages means with the same letter are not significantly different at P<0.05.

The presents or absence of *D. isaea* had no significant effect on the number of *L. bryoniae* mines, pupae or adults in either mature or juvenile plants (Figure 5-4).

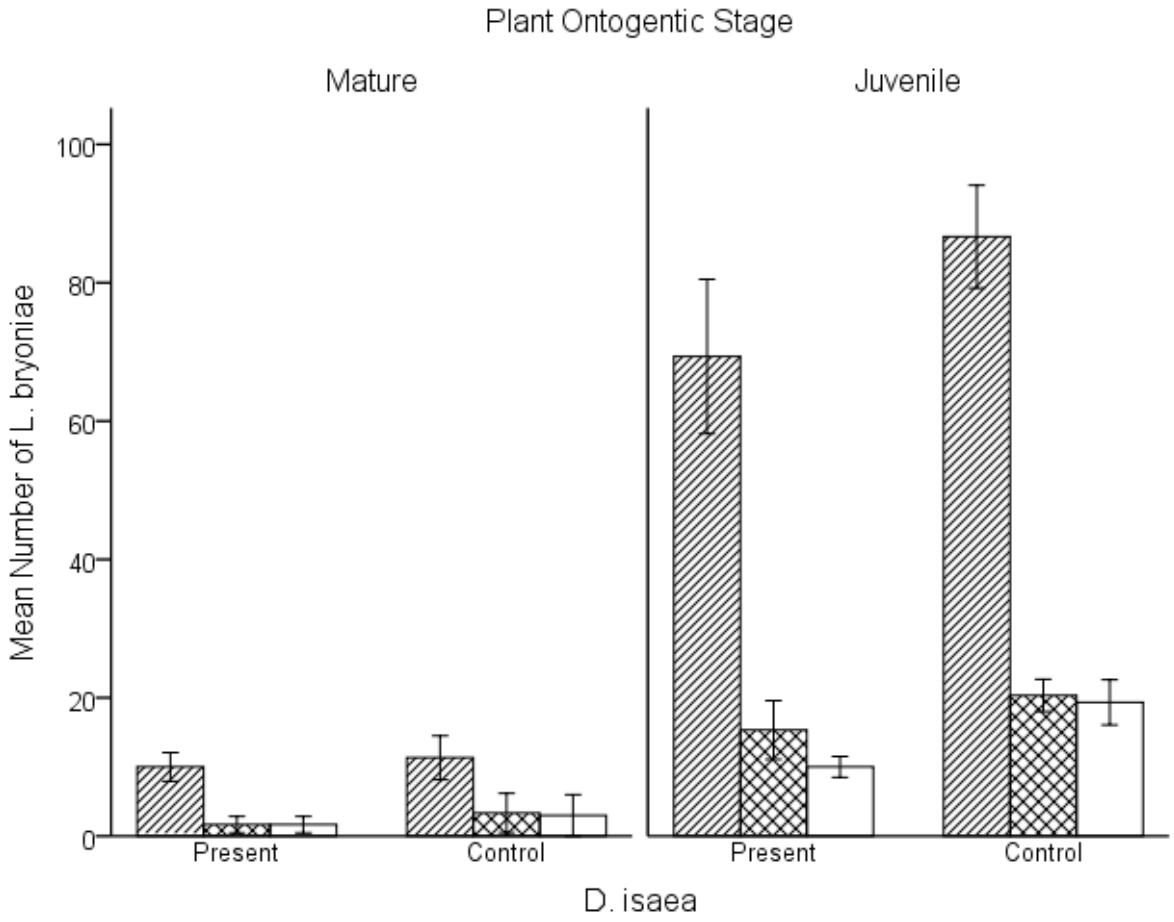

Figure 5-4: Mean ± standard error of the number of *L. bryoniae* mines (diagonal stripe), pupae (hashed) and adults (unpatterned) in the presence or absence (control) of the parasitoid *D. isaea* in mature and juvenile plants, with three replicates per treatment per ontogenetic stage. At $P<0.05$ *D. isaea* presents had no significant on the number of *L. bryoniae* mines, pupae or adults.

The wing length of *L. bryoniae* was significantly affected by both plant ontogenetic stage ($F_{(1,103)}=10.112$, $P=0.002$) and sex ($F_{(1,103)}=5.752$, $P=0.018$), with emerging adults from mature plants and females both having the greater wing lengths; however, neither generation, parental vs. offspring, nor any of the interaction terms were significant (Figure 5-5).

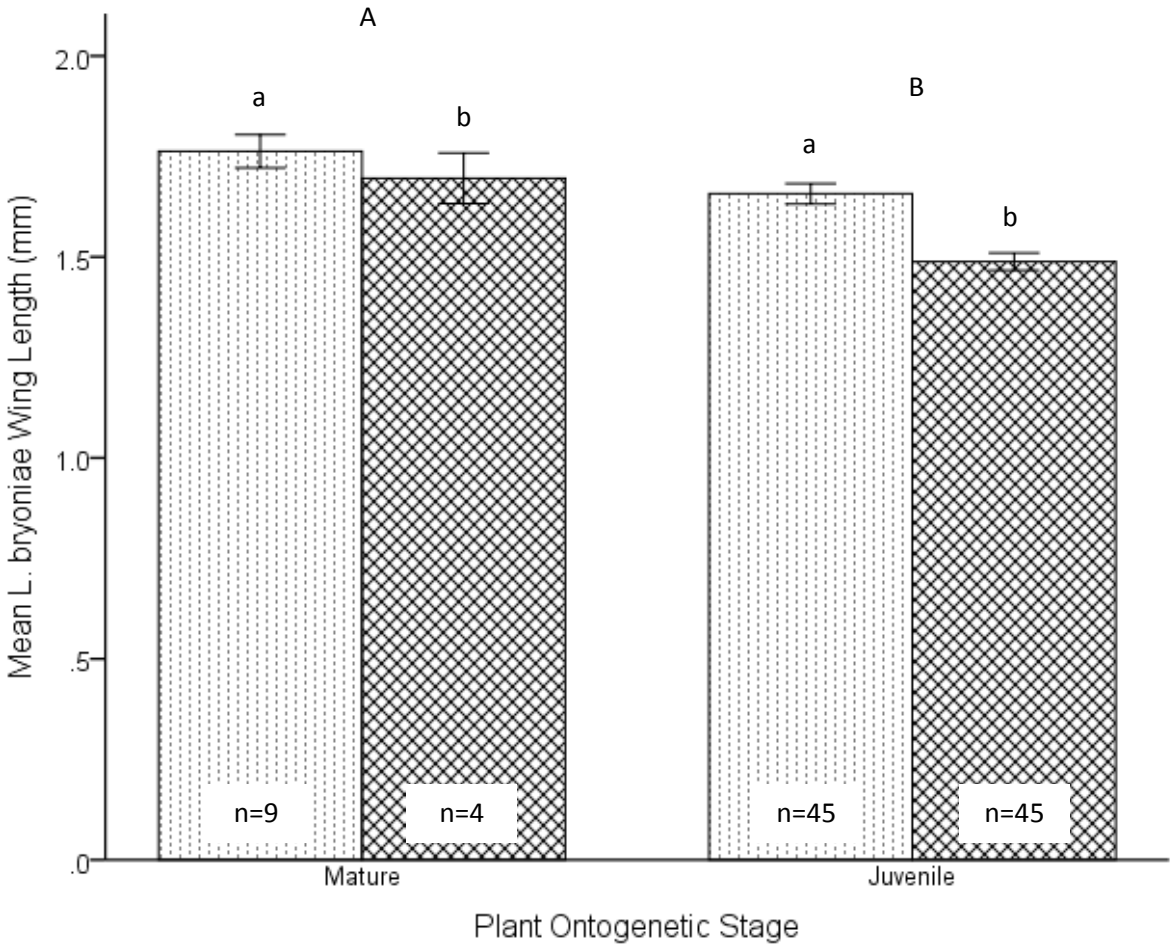

Figure 5-5: Mean ± standard error of the wing length (mm) of newly emerged female (dotted) and male (hashed) adult *L. bryoniae* originating from both mature and juvenile tomato plants. Means with the same letter (uppercase for between host plant ontogenetic stages and lowercase for between *L. bryoniae* genders within host plant ontogenetic stages) were not significantly different at $P<0.05$.

5.4.3. *Diglyphus isaea*

Diglyphus isaea emerged only from the juvenile plants, with a mean number of 16 adults emerging into each cage of small plants (Figure 5-6). A log-likelihood good of fit test (G-test) with William's correction factor revealed that there was no significant deviation from a gender ratio of 1:1, when significance levels were determined using χ^2 distributions at one degree of freedom (G=0.33, P=0.565).

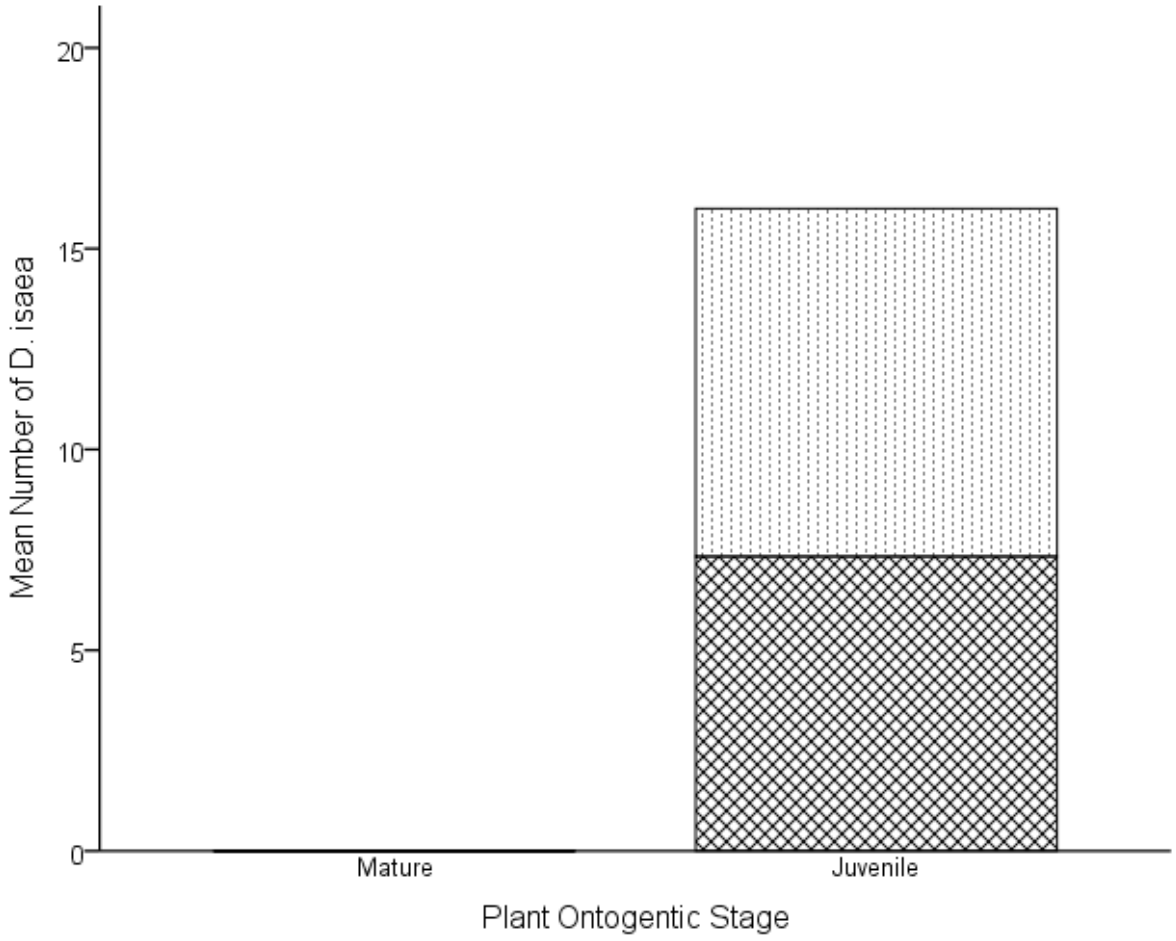

Figure 5-6: Mean number of male (hashed) and female (dotted) *D. isaea* emerging from cages of mature and juvenile *L. bryoniae* infested tomato plants, with three replicates per plant ontogenetic stage. At P<0.05 there was no significant deviation from the expected proportion of 0.5 males.

Two-Way ANOVA demonstrated that there was a significant difference in wing length between male and female *D. isaea* ($F_{(1,87)}$=27.507, P=0.000); however, there is no significant effect of generation nor was the interaction term significant (Figure 5-7).

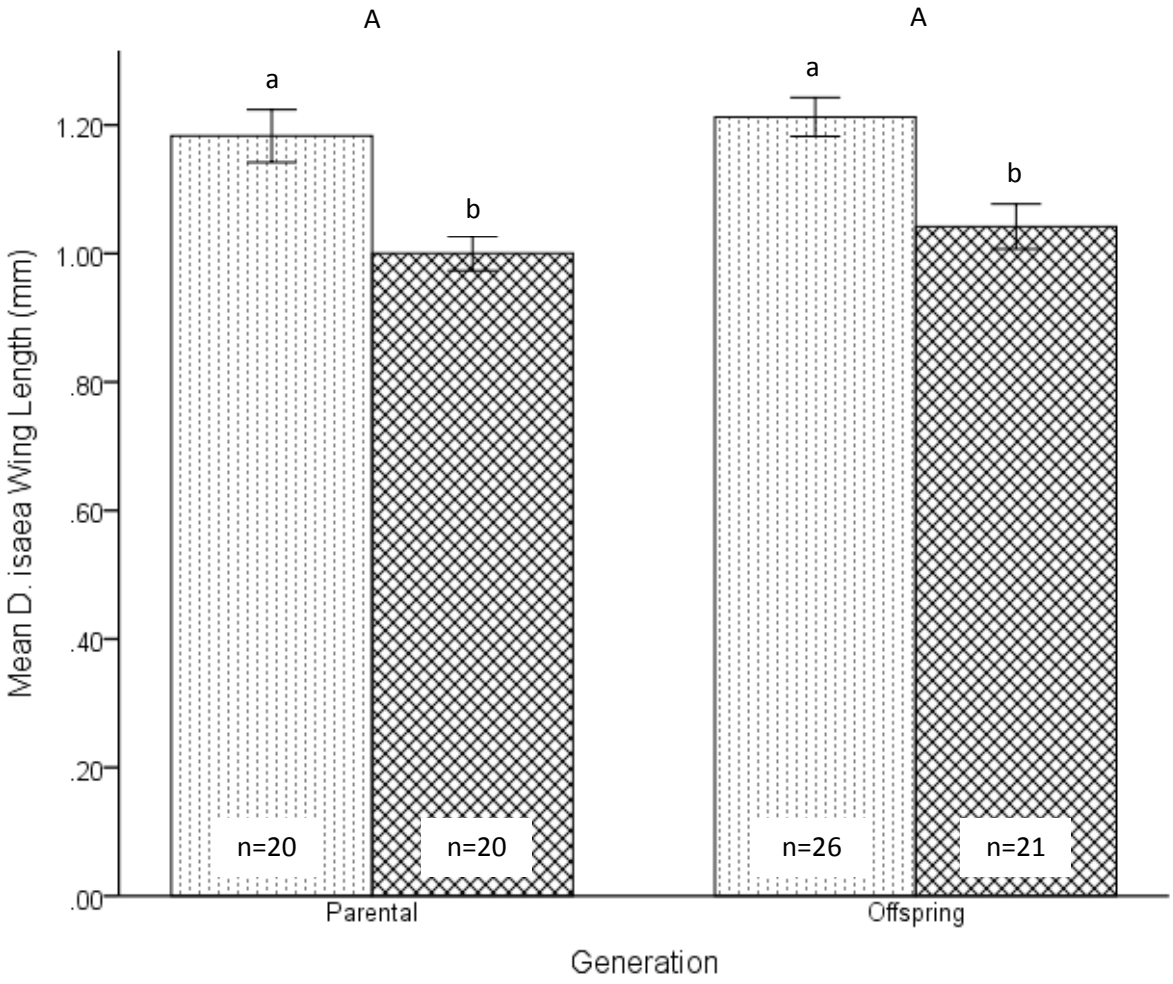

Figure 5-7: Mean ± standard error of the wing length of female (dotted) and male (hashed) of *D. isaea* from both the parental generation and the offspring generation. Means with the same letter (uppercase for between generations and lowercase for between genders within generation) are not significantly different at P<0.05.

5.5. Discussion

Juvenile plants were subjected to greater leaf-puncturing and more mining larvae than mature plants. Leaf punctures are formed by adult female leaf miners using their toothed ovipositor and can be made either in order to obtain leaf sap, for their own nutrition, or in order to oviposit into the leaf, from where newly hatched larvae will burrow into the mesophyll layer (Malais & Ravensberg, 2003). Increased leaf puncturing in younger plants may reflect the lower levels of physical defences present in these plants compared with their older counterparts; for example, glandular trichome densities are known to increase with age in the wild tomato *Lycopersicon hirsutum* (Leite *et al.*, 2001), and such defences may hinder *L. bryoniae* females in their attempt to feed and oviposit on the leaf surface in older plants. Leaf toughness may also increase with age, although, at least for *L. trofolii* on potato, this does not appear to prevent or inhibit oviposition on older leaves (Facknath, 2005).

In the current study leaf punctures were examined only by eye and the presence or absence of an egg in a puncture was therefore not recorded, so the relative ratios of feeding to oviposition leaf punctures in the juvenile or mature plants could not be determined. However, the proportion of leaf punctures to mines was lower in the juvenile plants with a ratio of 27.34 leaf punctures per mine in the juvenile plants compared with 61.62 leaf punctures per mine in the mature plants. This pattern of leaf punctures to mines suggests that oviposition was more frequent on the juvenile plants. As females of *Liriomyza* spp. are known to require proteins from their diet for egg maturation (Spencer 1973, cited in Minkenberg & van Lenteren, 1986) and the leaves of younger tomato plants are known to contain a higher proportion of nitrogen than the leaves of older plants (Minkenberg &

Ottenheim, 1990), this might indicate that fewer feeding punctures are required to obtain sufficient protein for oviposition. Alternatively, leaf traits such as size, colour and fluctuating asymmetry may influence the ovipositional preference of the female leaf miners (Ishino *et al.*, 2011).

An alternative explanation for the variation in the leaf puncture to mine ratio is that ovipostion is equal on the two set of plants but that egg stage mortality is higher in the mature plants. The constitutive element of chemical defence in plants is known to increase with age in herbaceous plants (Barton & Koricheva, 2010) and if *L. bryoniae* eggs are vulnerable to tomato plant defences, higher egg stage mortality would be expected in the larger plants.

Larval to adult survival also varied marginally with the ontogentic stage of the host plant, with higher survival (4.58 mines per adult) in the mature plants than in the juvenile plants (5.32 mines per adult). Although this is contrary to what may be expected from the nutritional composition of leaves, as the more mature leaves have a lower nitrogen content (Minkenberg & Ottenheim, 1990), it can be explained both by density dependent competition, which will have been considerably stronger among the larvae in the juvenile plants, and a quality (juvenile plants) vs. quantity (mature plants) trade-off (Eber, 2004). This is supported by the observation that *L. bryoniae* from mature plants are larger than those from juvenile plants (see Figure 5-5), suggesting that for individuals, quantity may be better than quality. This pattern of increased survival in mature plants is contrary to the assertion that the constitutive element of chemical defence increase with age in herbaceous plants; however, it has be suggested that secondary chemical defences could be ineffective

against larval leaf miners as the selective consumption of specific tissues within the leaf may allow the avoidance of toxic compounds (Connor & Taverner, 1997).

In this study *D. isaea* did not parasitise *L. bryoniae* larvae in mature tomato plants, and although the total absence of parasitism is surprising, it can be explained in a number of ways. Firstly, the increase of glandular trichome densities (Leite *et al.*, 2001) and leaf toughness (Facknath, 2005) with age has been observed in other members of the Solanaceae family and may inhibit *D. isaea* females attempting to parasitise *L. bryoniae* leaf miners through the surface of the leaf. Secondly, ontogentic stage has been shown to effect the production of indirect defences (e.g. HIPVs) (Boege & Marquis, 2005) which matches the general pattern for herbaceous plants, that induced chemical defences should decrease with maturity (Barton & Koricheva, 2010). Hare (2010) has shown that the perennial Sacred Datura, *Datura wrightii*, which is also a member of the Solanaceae family, shows seasonal variation in HIPV production in response to two pests, *Lema daturaphila* and *Tupiocoris notatus*, with highest production occurring during the vegetative growth phase which declines after the onset of flowering. If tomato follows a similar pattern of HIPV production through its ontogentic stages then it is likely that the mature plants may not emit sufficient volatiles to induce a response from *D. isaea* females.

Although female *D. isaea* can reduce leaf miner numbers both through parasitism and host feeding, the presence of *D. isaea* did not have a significant affect on *L. bryoniae* numbers (Figure 5-4), suggesting that in the system examined in this study 'bottom-up' regulation, e.g. plant quality and defence, may be more important in determining the *L. bryoniae* population size and individual survival than 'top-down' regulation, e.g. predation. However,

although insignificant, there is a consistent decrease in *L. bryoniae* populations in the presence of *D. isaea* which suggests that a higher ratio of *D. isaea* adults to *L. bryoniae* mines may have a stronger effect. Determining the optimal release rate of a control agent is essential for effective and economic biological control; however, it is apparent that in the current study insufficient *D. isaea* were used to have an impact on *L. bryoniae* numbers.

In conclusion, juvenile plants are more vulnerable to *L. bryoniae* attack both in terms of leaf puncturing and oviposition. However, whilst juvenile plants may support a greater number of *L. bryoniae*, those individuals which developed on mature plants attained a larger size. A new generation of *D. isaea* was formed only on the juvenile plants; however, under current leaf miner control regimes *D. isaea* is used only later in the season when the endoprasitoid *Dacnusa sibirica* is no longer efficient (pers. comm. J. Klapwijk), by this point in the season the plants have reached maturity, the current study suggests *D. isaea* may not be an effective control agent against *L. bryoniae* under these conditions. Further to which at the release rate used there was no significant effect of the presence of *D. isaea* on the number of *L. bryoniae* in juvenile plants, which may also be of concern to biological control practitioners.

6. *DIGLYPHUS ISAEA* HOST LOCATION: Y-TUBE OLFACTOMETERY AND TRAP ASSAYS.

6.1. Abstract

The cocktail of volatile chemicals emitted by plants which are under attack by insect herbivores is commonly used by parasitoids to locate potential hosts. It is likely that parasitoids learn the cues that will lead to a host from their own natal environment. The biological control agent *Diglyphus isaea* is mass reared on leaf miner infested French bean plants but it is commonly used against this pest in other crops, such as tomato. Trap assay and Y-tube olfactometery were used to examine the use of plant volatiles and the effects of natal host on *D. isaea* host location behaviour. No preference for *Liriomyza bryoniae* infested or uninfested French bean leaves was found nor any effect of natal host on preferences for natal host vs. novel *L. bryoniae* infested plants. This apparent ambivalence for herbivore induced plant volatiles is discussed.

6.2. Introduction

Part of the defensive strategy used by plants to protect themselves from herbivorous insects is to emit a cocktail of volatile chemicals which signal to insect predators and parasitoids that there are herbivores present (Dicke *et al.*, 1990; Takabayashi & Dicke, 1996; Turlings *et al.*, 1995). These herbivore-induced plant volatiles (HIPVs) are known to be specific to both the species of plant under attack and the species of herbivore making the attack; this specificity allows predators and parasitoids to accurately locate suitable prey or hosts (Turlings *et al.*,

1993; Wei, Zhu, & Kang, 2006). A long running debate in insect ecology has focused on how such natural enemy species, especially generalists, might acquire the knowledge that allows them to identify the cues, including HIPVs, which can lead them to potential prey or hosts.

Hopkins' host selection principle is based around the observation that as adults, many insect herbivores and parasitoids prefer to feed and oviposit on the same species on which they had themselves developed (Barron, 2001). This preference has been explained in three ways: as a genetic inherited preference, through preimaginal conditioning to host cues, and through imaginal conditioning to host cues. Any genetic element influencing host preference can be explained in terms of biotypes or host races (see Diehl & Bush, 1984). The concept of preimaginal conditioning, suggested by Thorpe and Jones (1937), has proved highly controversial due to the large scale reorganisation of the central nervous system during metamorphosis in holometabolous insects (Barron, 2001). However, Pavlovian-style conditioning experiments have been used to demonstrate the possibility of preimaginal conditioning, at least under certain contrived conditions (Blackiston, Casey, & Weiss, 2008; Tully, Cambiazo, & Kruse, 1994). The possibility that conditioning to cues from the natal host takes place in newly emerged adults has been more favourably received, and is generally attributed to cues from the larval environment present on the surface of the pupal case influencing the preference of the newly emerged adult (Barron & Corbet, 1999; Herdard *et al.*, 1988). Chemical legacy hypothesis takes this idea further, suggesting that the apparent retention of associative learning through metamorphosis is not due to the survival of intact synaptic connections but that minute particles from the larval habitat present within the insect's body influence its adult preferences (Corbet, 1985). Through one or more of these

routes it is possible that a parasitoid may become conditioned to cues, such as HIPVs, from its larval host-plant complex, which in adult life influence its host searching behaviours.

Diglyphus isaea is an ectoparasitod of leaf mining diptera. Adult females parasitise larval leaf miners whilst they are actively feeding within a plant's leaf; the young parasitoid also remains within the leaf throughout the larval and pupal phases only emerging from the leaf as an adult (Malais & Ravensberg, 2003). *Diglyphus isaea* is commercially available as a biological control agent for use against *Liriomyza spp.* (http://www.koppert.com). For the purpose of mass production *D. isaea* is reared on *Liriomyza spp.* infested French bean, *Phaseolus vulgaris*. However, as it is more commonly used by growers against *Liriomyza spp*. in tomato, *Solanum lycopersicon,* concern has been raised about the efficacy of *D. isaea* in this crop (pers. com. J. Klapwijk). Previous chapters have examined the effect of host plant on *D. isaea* productivity in terms of the number of offspring produced and the reduction in *L. bryoniae* numbers. This chapter examines whether *D. isaea* utilise HIPVs for host location and how the culturing host may affect the preference expressed by *D. isaea* for the volatiles emitted by bean or tomato plants.

6.3. Materials and Methods

6.3.1. Y-tube olfactometer

6.3.1.1. Insects

Diglyphus isaea were reared according to the standard protocol (see Chapter 2) on the conventional host plant French bean but also for one generation on the novel host plant tomato.

6.3.1.2. The olfactometer

To examine how natal host plant affects the preferences of parasitoids to a variety of odour cues, individual parasitoids were assessed in a Y-tube olfactometer. The olfactometer was formed from clear plastic tubing with an internal diameter of 22 mm and consisted of a central trunk 75 mm long from one end of which there were two 65 mm long branches each at a 145° angle to the main trunk, forming a Y-shape; these branches were closed at the terminal end. A single AVMINI aquarium air pump (Interpet; U.K.) provided a 75 lt $hour^{-1}$ flow of air which passed through a 6mm silicon tube; the silicon tube branched in two and the two columns of air passed through separate odour cue chambers before entering the terminal ends of the Y-tube olfactometer branches (Figure 6-1). In this way separate odour cues could be introduced to each branch of the Y-tube olfactometer at an identical rate of air flow. A parasitoid was introduced to the olfactometer through the open end of the trunk, which was then covered with a section of polyester netting (96 x 26 mesh) to prevent the parasitoid escaping, whilst allowing air flow through the apparatus; similarly, netting was used to prevent parasitoids from entering the silicon tubes. The Y-tube olfactometer was placed on a board at a 40° angle within a box with the branching ends uppermost, and

illuminated from above with a halogen lamp (SCHOTT KP 750, Germany). The purpose of this illumination was to take advantage of the parasitoids' positively phototactic behaviour (Chiel *et al.* 2006) and to encourage the parasitoid to move up the trunk of the olfactometer and enter the branches.

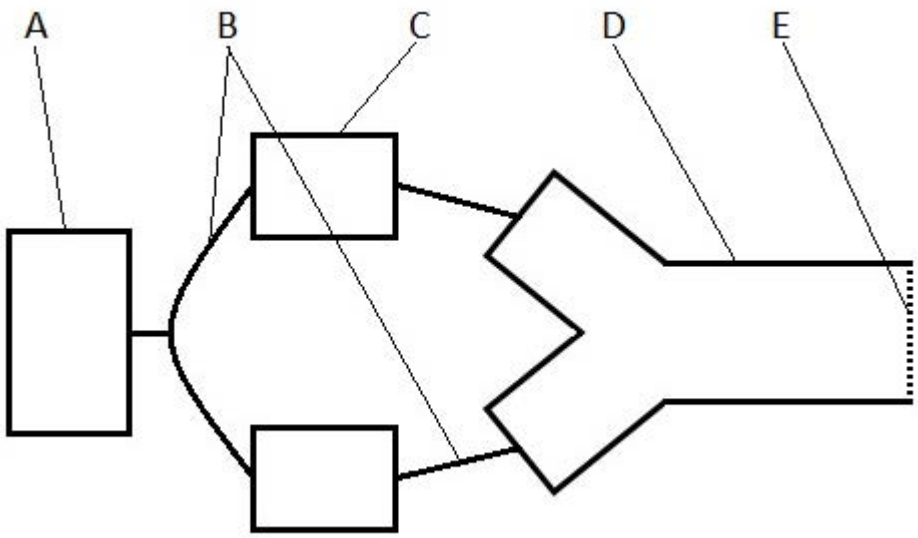

Figure 6-1: Design of the Y-tube olfactometer, showing: A) air flow provided by an aquarium air pump; B) is forced through silicon tubing; C) over odour cues in two small chambers; D) before entering the two branching ends of the Y-tube; E) parasitoids can be inserted through the open end of the trunk which is then closed with a mesh cap.

6.3.1.3. The Y-tube assay

Newly eclosed female *D. isaea* were placed individually into the Y-tube olfactometer. The insects were tested individually to prevent any bias which may have been cause by the behaviour of one insect influencing another. As only the largest parasitoids were able to move against the air flow, a method of pulsing the air flow was therefore adopted, where by the pump was turned on for one minute and then switched off for five minutes, and this

cycle was repeated twice. A parsitoid was deemed to have made its choice once it had moved 1.5cm into one of the branches; both the branch chosen and the time taken to make that choice were recorded. If a parasitoid had failed to respond after two cycles of air pulsing (12 minutes) it was assumed to be a none responder and was removed from the olfactometer. Three odour sources, *L. bryoniae* infested French bean (*Phaseolus vulgaris* L., var. Tendergreen), *L. bryoniae* infested tomato (*Solanum lycopersicon* L. (also known as *Lycopersicon esculentum Mill.), var. Elanto rz*) and a blank (no odour), were used in three combinations: French bean vs. blank, tomato vs. blank and French bean vs. tomato. To provide the infested French bean and tomato odours a $2cm^2$ segment of leaf containing a single 2^{nd} or 3^{rd} instar *L. bryoniae* larva and a section of mine was used. The leaf segments were replaced regularly, after every five to ten paristoids tested, and the branch through which odours entered the Y-tube olfactometer (e.g. left or right) was also swapped between each set of parasitoids to ensure that there was no directional bias. Between odour combinations, when swapping the arm through which an odour entered the Y-tube olfactometer, and at the end of every day, the Y-tube olfactometer, silicon tubing and odour cue chambers where emptied, washed and oven dried.

6.3.2. Trap assays

The trap assay method was originally designed by Woodard *et al.* (1989) for the purpose of segregating *Drosophila melanogaster* mutants with olfactory system defects from their wild type counterparts. A similar method has been utilised by Carlson (1996) for the same purpose but a modified method was developed by Barron & Corbet (1999) to quantify the response of *D. melanogaster* reared in the presence of varying levels of methanol to methanol odour cues. The assay used here is in structure most similar to that of Barron &

Corbet (1999), but is used to quantify the response of *D. isaea* reared on *L. bryoniae* in French bean to *L. bryoniae* infested and uninfested French bean leaves.

Traps were made from a 30ml Universal tube, a 7ml BIJOU tube, a yellow 200ul pipette tip and polyester netting (96 x 26 mesh). The closed end of the Universal tube was cut off and the resulting opening was covered with mesh to allow ventilation without allowing insects to escape. The pipette tip was cut down to a 20 mm length so there was aperture of 5mm internal diameter at one end and a narrower 3 mm aperture at the other end. A 5mm diameter hole was cut through the lid of the BIJOU tube and the narrower end of the pipette tip was inserted into the hole and the tip was pushed through until it filled the hole and was firmly wedged. The BIJOU tube was then inserted into the open end of the Universal tube, in a tight fit, so the final structure consisted of a large chamber linked to a smaller chamber by a narrow passage (Figure 6-2).

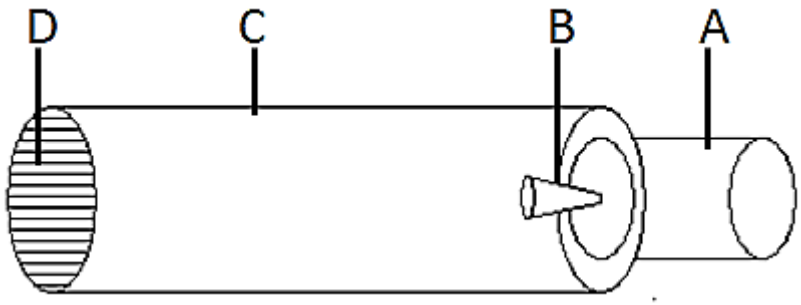

Figure 6-2: Schematic diagram of the trap assay system: formed from a 7ml BIJOU tube (A), a 200ul pipette tip (B), a 30ml Universal tube (C) and Polyester netting (D). The pipette tip (B) allowed odours to diffuse from the smaller chamber (A) to the larger chamber (C) and for insects to move between the two chambers.

The assay was operated by placing an odour cue into the smaller chamber and introducing four two day post-eclosion unfed female *D. isaea* into the larger chamber and observing the time taken for the first insect to move from the larger to the smaller chamber. Only unfed females two days post-eclosion were used; firstly, to ensure a response was elicited, secondly, because at the temperature the insects were reared, the pre-oviposition period is approximately two days (Minkenberg, 1989), and thirdly, because adult males do not feed or oviposit (Malais & Ravensberg, 2003) so may not respond to plant odours or HIPVs in the same ways as females. Four insects were used in each assay to ensure (as far as possible) that there was at least one which moved during a six hour period. Only the time taken by the first insect to move was recorded as the movement of any subsequent insects may have been influenced by the first. Odour cues consisted of either: a $2cm^2$ segment of French bean leaf containing a single 2^{nd} or 3^{rd} instar *L. bryoniae* larva and a section of mine, or a $2cm^2$ segment of French bean leaf from a healthy uninfested plant.

6.3.3. Statistical analysis

Log-likelihood goodness-of-fit tests, with Williams' correction factor (see section 3.3.8), were used to examine whether parasitoids from either host plant exhibit any preference between the two odour cues in each combination, with significance levels determined using χ^2 distributions at one degree of freedom. One and Two-way ANOVAs were used as appropriate to analyse the number of parasitoids responding to the odour cues and the time taken for a response to be elicited in both the Y-tube olfactometer and the trap assay.

6.4. Results

6.4.1. Y-tube olfactometer

A total of 180 parasitoids were tested with 30 replicates for each host plant-odour cue combination. Of these, however, nearly one third (55 parasitoids) showed no response and remained in the trunk of the olfactometer throughout two cycles of air flow pulsing. The *D. isaea* used were reared on *L. bryoniae* either in conventional plant host French bean or in the novel plant host tomato. However, Log-likelihood goodness-of-fit tests have shown that neither those parasitoids reared on French bean, nor those reared on tomato exhibited a preference for any of the odour cues. A two-way ANOVA confirms that there is no difference in the number of parasiotids responding either between the two groups of parasioids or the between the three odour cue combinations (Figure 6-3).

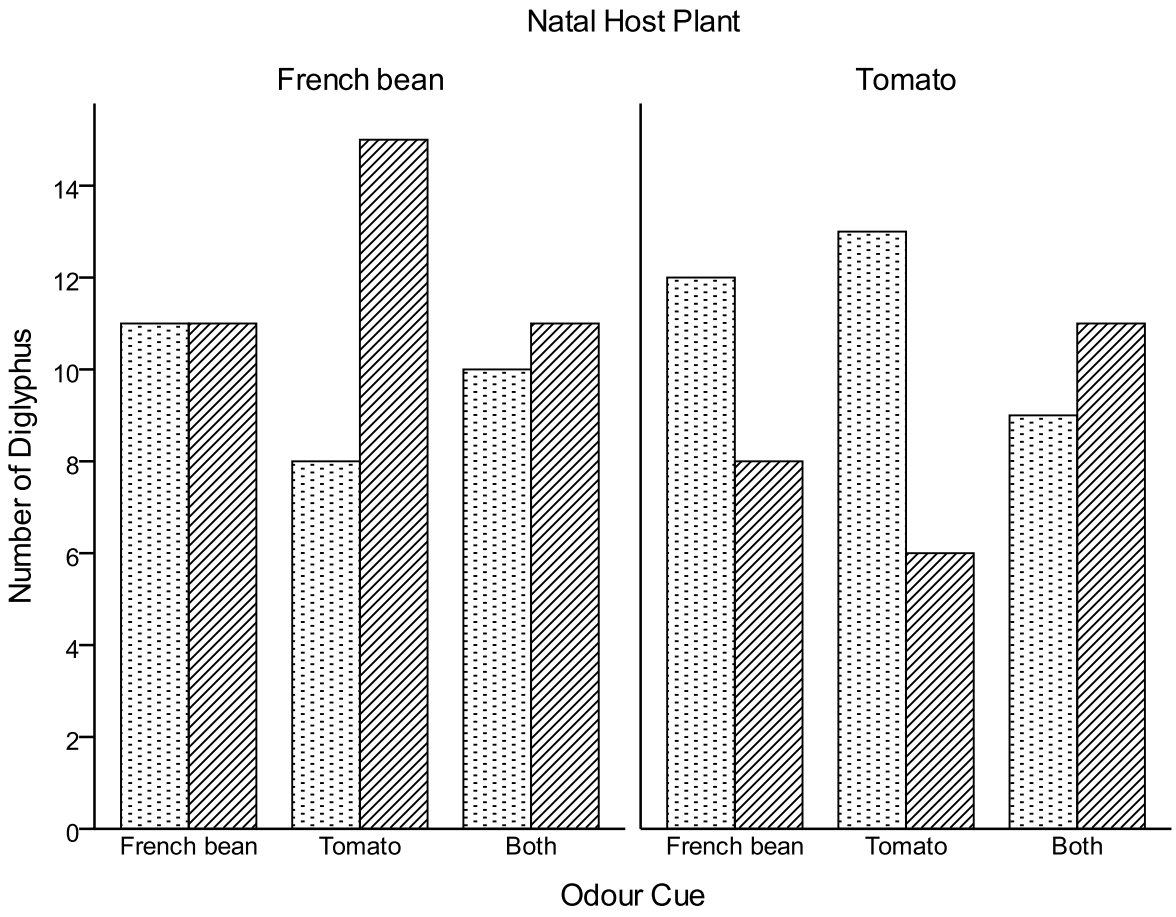

Figure 6-3: Number of *D. isaea* responding to each odour cue: *L. bryoniae* infested 'French bean' (dotted) vs. blank (diagonal stripe), *L. bryoniae* infested 'Tomato' (dotted) vs. blank (diagonal stripe), and 'Both', *L. bryoniae* infested French bean (dotted) vs. *L. bryoniae* infested tomato (diagonal stripe), for *D. isaea* reared on *L. bryoniae* in the conventional host plant French bean and on the novel host plant tomato, with thirty replicates per treatment. Neither parasitoid population showed any significant preference for any odour cue at $P<0.05$.

Analysis of the time taken by a parasitoid to choose between the arms of the olfactometer (Figure 6-4) revealed that within each pair of cues (e.g. *L. bryoniae* infested French been vs.

blank), there was no difference in time taken regardless of the choice made. Between the cue pairs, comparison of the decision making time of French bean-reared *D. isaea* in the *L. bryoniae* infested French been vs. balnk and the *L. bryoniae* infested tomato vs. blank assays, *D. isaea* moved significantly more quickly to the arm containing the odour of its natal host than towards the arm containing a novel host ($F=7.132$, d.f.=1 $P=0.016$). However, tomato-reared *D. isaea* in the same two assays showed no difference in decision making time. Regardless of the choice made when presented with a choice of *L. bryoniae* infested French bean vs. *L. bryoniae* infested tomato, tomato-reared *D. isaea* show a significantly shorter decision making time than the conventionally reared *D. isaea* ($F=6.149$, d.f.=1, $P=0.018$).

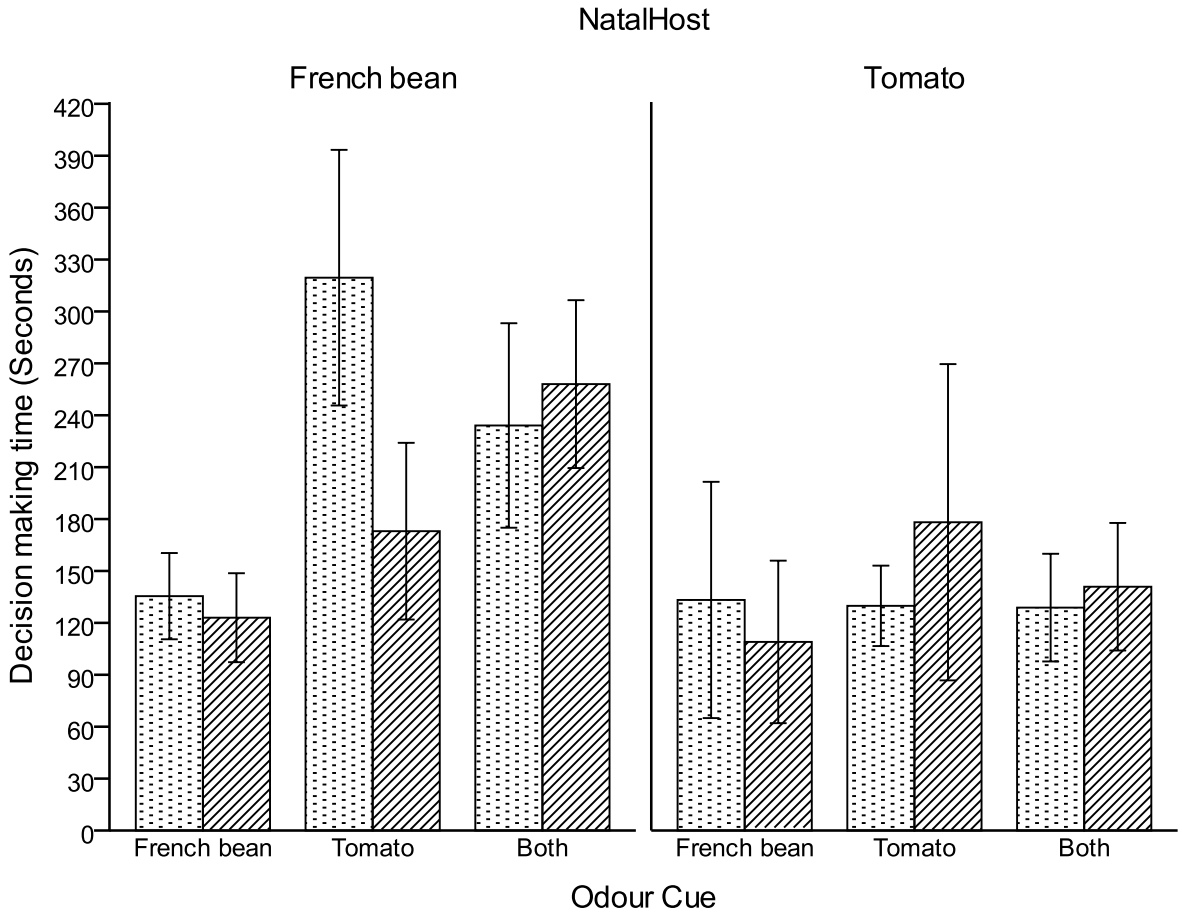

Figure 6-4: Mean ± standard error of the decision making time taken in seconds for *D. isaea* reared on *L. bryoniae* in the conventional plant host French bean and the novel plant host tomato, when presented with pairs of odour cues: *L. bryoniae* infested 'French bean' (dotted) vs. blank (dashed), *L. bryoniae* infested 'Tomato' (dotted) vs. blank (dashed), and 'Both' *L. bryoniae* infested French bean (dotted) vs. *L. bryoniae* infested tomato (dashed), in a Y-tube olfactometer, with thirty replicates per treatment.

6.4.2. Trap assays

Trap assays were used to test for any preference between the odours emitted from *L. bryoniae* infested French bean leaf segments and uninfested leaf segments in the

conventionally reared *D. isaea*. Eighty *D. isaea* females were used to achieve 10 replicates with each odour cue and in every case at least one individual moved from the main chamber of the assay equipment into the trap within the six hour observation period. However, *Diglyphus isaea* females show no difference in the time taken to respond to the odour of uninfested or infested leaves (Figure 6-5), ($F_{(1,18)}$1.798, P=0.197).

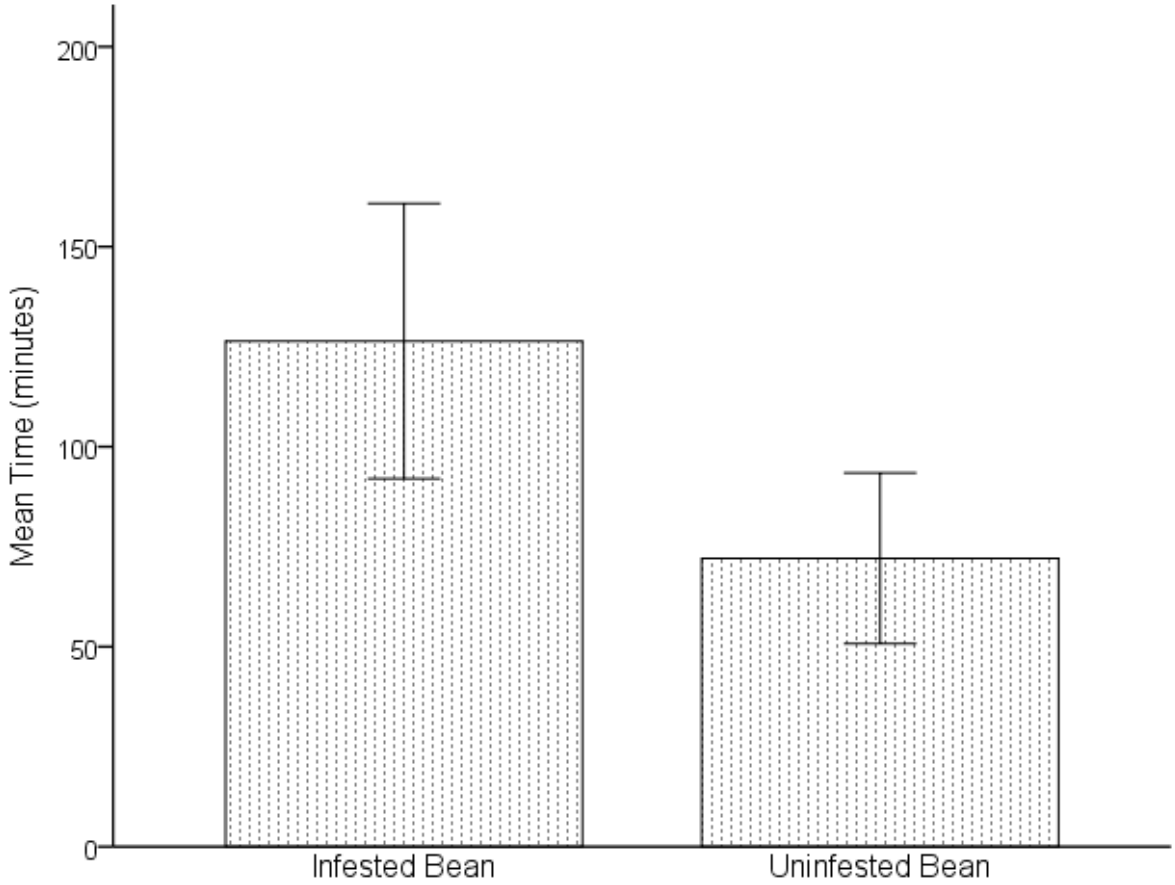

Figure 6-5: Mean ± standard error of the time (minutes) taken by female *D. isaea* to respond to the odour of *L. bryoniae* infested and uninfested leaf segments by moving through the trap assay apparatus, ten replicates per odour cue. At P<0.05 no significant preference was shown by *D. isaea* for either odour cue.

6.5. Discussion

The use of HIPVs as a means of long range host location is generally considered ubiquitous among parasitoids (Bjorksten & Hoffmann, 1998; Hedard *et al.*, 1988; Kang *et al.*, 2009; Zhao & Kang, 2002). However, the results of the present study put this widely held belief into question, as there is little evidence for *D. isaea* showing any response to HIVPs, though there are several possible explanations for this lack of response:

In the current study, as in previous investigations on the response of *D. isaea* to plant volatiles (Finidori-Logli *et al.*, 1996; Zhao & Kang, 2002), the odour source used was part rather than the whole of a plant and whilst it is known that plants release a specific cocktail of HIPVs in response to attack by leaf miners, it is also known that plants produce a quantitatively different cocktail of volatiles in response to mechanical damage (Wei *et al.*, 2006). The cocktail of volatiles produced by mechanically-damaged plants contains a much higher concentration of Green Leaf Volatiles (GLVs) than leaf miner damaged plants (Visser, 1979; Wei *et al.*, 2006; Zhao & Kang, 2002). Zhao & Kang (2002) used electroantennograms to demonstrate than *D. isaea* antenne are responsive to mechanically-damaged plants and hypothesised that the high concentrations of GLVs in the mechanically-damaged leaves were responsible for this. It is likely that this provides an explanation for the lack of discernment between the two cues used in the trap assay experiment, as both the infested and uninfested leaf segments were mechanically-damaged and therefore emitting high concentrations of GLVs.

Whilst broad differences in HIVPs may exist between mechanically-damaged and herbivore-infested plant materials, the cocktail HIVPs released by plants are also known to be highly

specific to both the species of plant under attack and the species of herbivore. It is also known that at least some species of parasitoid are able to detect these differences in HIPVs (Turlings *et al.*, 1993; Wei, Zhu, & Kang, 2006). It is possible that HIPVs also vary more subtly with factors such as seasonal growth/senescence (Hare, 2010), the ontogenetic stage of the plant (Rostás & Eggert, 2007), the variety or cultivar of the plant (Eber, 2004; Hoballah *et al.*, 2002), the larval instar of the herbivore (Gouinguené, Alborn, & Turling, 2003) and the local environmental conditions (Gouinguene & Turlings, 2007; Schmelz *et al.*, 2003). At the species level *D. isaea* is known to feed on a number of species of polyphagous leaf-mining flies and at least one species of leaf-mining lepidopteran (Boucek & Askew 1968, cited in Minkenberg & van Lenteren, 1986). With so much potential variation in the cocktails of HIPVs that *D. isaea* might detect, the question arises, could it be possible for *D. isaea* to use HIPVs to find potential hosts? Zhao & Kang (2002) demonstrated that *D. isaea* can exhibit an electroantennogram response to both bean and tomato leaves infested with *L. sativae*; however, this does not demonstrate a link between antennal stimulation and a behavioural response to HIPVs.

The volatiles emitted by a plant change both with plant age and seasonally (Hare, 2010; Rostás & Eggert, 2007). In the current study the *D. isaea* adults used had emerged from older plants than those offered to them in either of the experiments. Therefore, if the learning of natal host plant cues occurs during the early imaginal period, as many authors argue (Barron & Corbet, 1999; Liu & Liu, 2006; van Emden *et al.*, 1996), then the *D. isaea* adults may have learnt a different set of cues to those emitted by the younger leaves used as odour sources in the two experiments, reducing their responsiveness to the cues provided.

It is, however, possible that *D. isaea* does not use HIPVs as a cue in order to locate plants which may be harbouring potential hosts. There are other possible sensory modes which *D. isaea* could use for host location: visual (Adriana Salvo & Valladares, 2004), auditory (Sugimoto *et al.*, 1988a; Sugimoto *et al.*, 1988b) and vibratory (Meyhöfer & Casas, 1999). Although Cheah & Coaker (1992) have demonstrated that *D. isaea* is capable of host locating in the dark, the visible presence of mines on the leaf surface has been suggested as a highly conspicuous cue which could be utilised for host location by leaf miner parasitoids (Kato, 1984). To date, few studies have examined *D. isaea*'s visual foraging, however, Chiel *et al.*, (2006) found that UV-filtered light did not inhibit host location. Alternatively, *D. isaea* may require multiple stimuli simultaneously in order to elicit a response.

In conclusion, the use of leaf segments rather than whole plants may have altered the volatile cocktail to which the *D. isaea* were exposed and this may have altered the preferences they displayed. Previously other authors have shown through electroantennagrams that *D. isaea* is able to detect GLVs, although this ability does not appear to manifest itself as a behavioural response in the current study, suggesting that plant volatlies may not play a major role in *D. isaea* host location.

7. THERMAL ECOLOGY OF *LIRIOMYZA BRYONIAE* AND *DIGLYPHUS ISAEA*.

7.1. Abstract

The thermal tolerances of an insect are described not only by those temperatures which are directly lethal but also by a subset of temperatures which limit normal behaviour, including both lower and upper thermal limits to coordinated movement and coma inducing temperatures. For parasitoids and predators used in biological control it is desirable that they should maintain coordinated movement at temperatures above and below those that cause their prey to be immobilised. However, theories on linking insect body size to thermal tolerance suggest that smaller insects should be more cold tolerant and larger insects more heat tolerant. The parasitoid *Diglyphus isaea* displays greater thermal tolerance (limits to coordinated movement 0.2°C and 42.0°C) than its host *Liriomyza bryoniae* (limits to coordinated movement 2.4°C and 41.3°). These results are discussed both in terms of the relative size of the insects and also with regard to the efficacy of *D. isaea* as a biological control agent of *L. bryoniae*.

7.2. Introduction

Temperature has been described as ecologically the most important environmental factor affecting life on earth (Cossins & Bowler, 1987), and this is particularly true for insects as their body temperature closely matches that of their environment (May, 1979; Stevenson,

1985). An insect's thermal tolerance therefore limits its distribution (Parmesan, 1996) and consequently it may be tempting to assume that geographically co-occurring insects will have the same thermal tolerance. However, a number of factors may affect this, such as differential use of shelters and resources or differences in phenology (Hoffmann, Sørensen, & Loeschcke, 2003; Le Lann *et al.*, 2011). An insect's thermal tolerance is not described only by the extreme high and extreme low temperatures which are directly lethal, as within the range of temperature at which an insect can live, there is a narrower range of temperatures within which an insect can exhibit normal behaviour, e.g. feed, reproduce, escape predation etc. (Mellanby, 1939). Non-lethal measures of insect thermal tolerance include the Critical Thermal Minimum (CTmin), Chill Coma Temperature (CCT), Critical Thermal Maximum (CTmax) and Heat Coma Temperature (HCT). Historically, an array of different definitions have been used to describe critical thermal and coma states (for a review see Hazell & Bale, 2011); however, contemporary authors have defined the critical thermal temperature as that at which an insect is no longer able to move in a coordinated manner (Sinclair *et al.*, 2006) and a coma state as complete immobility identified as the temperature at which the 'last twitch' of an appendage (leg, wing or antenna) occurs (Hughes *et al.,* 2010).

Size is often cited as a critical factor determining an insect's thermal tolerance; smaller insects which have a higher surface-area to volume ratio are generally considered to be more resistant to low temperatures (Renault *et al.*, 2003) due to lower water content, promoting higher concentrations of solutes in body fluids which depresses the freezing temperature of the insect (Le Lann *et al.*, 2011; Renault *et al.*, 2003; Storey & Storey, 1992). By comparison, a larger size (lower surface-area to volume ratio) is assumed to be correlated

with increased water content and therefore increasing the insects resistance to dehydration (Block, 1996; Duncan & Lighton, 1994).

In an ecological context it would be beneficial to a predator or parasitoid if it were to have a greater tolerance to both extreme high and extreme low temperatures than its prey or host. This is also true in biological control where for maximum efficacy a biocontrol agent would be required to have a broader resistance than the target pest in order to provide effective control both early in the season, when ambient temperatures are still low and young plants are at their most vulnerable to pests, and at the height of summer when peak temperatures coincide with economically important milestones such as fruit set or maturation. However, parasitoids are invariably smaller than their hosts, suggesting that despite the obvious benefits to the parasitoid of maintaining coordinated locomotion at temperatures above those at which their host becomes immobile, this may not occur.

As the aim of augmentative biological control is to establish a self-sustaining population of control agents, lasting for a least one season or longer in some ornamental crops, it is important to determine the thermal tolerance of the most vulnerable life stage/s of a predator or parasitoid. Generally the pre-adult stages of parasitods are considered to be most vulnerable as their limited mobility prevents escape from unfavourable conditions (Feder, Blair, & Figueras, 1997); however, egg and pupal stages can be highly resistant to extreme temperatures (Norry & Loeschcke, 2002). In the cases of *L. bryoniae* and *D. isaea* the pre-adult life stages occur inside the leaf of the host plant where an element of thermal buffering protects the pre-adult insects from extreme temperatures (Pincebourde & Casas, 2006); adults, however, are free living without any form of protection, except of course in

glasshouses, and may therefore be subject to thermal stresses. This study will, therefore, assess the thermal activity thresholds of adult *D. isaea* and adults of its host species *L. bryoniae*, as the non-sedentary pre-adult stages of both species occur within the tissue of a plant leaf. These data are discussed in relation to the efficacy of *D. isaea* as a biological control agent and how this may vary seasonally, and more generally, the relationship between an insect's size and its thermal activity thresholds is also discussed.

7.3. Materials and Methods

7.3.1. Culturing of insect material

The origins and culturing of both *L. bryoniae* and *D. isaea* are as described in Chapter 2. The individual insects used in this study were collected as unfed newly emerged adults and their thermal activity thresholds were assessed within 12 hours of eclosion.

7.3.2. Experimental system

Lower (CTmin and CCT) and upper (CTmax and HCT) thermal activity thresholds of *L. bryoniae* and *D. isaea* were assessed using the insect arena design described by Hazell *et al.* (2008). This system consists of an aluminium block (Figure 7-1) into which a circular depression, the "arena", has been milled, and a network of channels through the block allows heated/cooled alcohol fluid to be pumped from a ramping alcohol bath (Haake Phoenix 11 P2, Thermo Electron Corp, Germany) through the block, thus controlling the temperature of the arena. A sample of between five and ten insects were placed into the arena which was covered with a thin sheet of clear Perspex, and the system was then heated or cooled at a rate of 0.2°C min^{-1} from the culturing temperature to either 50°C or -10°C

respectively. Insect activity was recorded using a digital video camera (Infinity 1-1; Lumenera Scientific, Canada) with a macro lens (Computa MLH-10X, CBC Corp, New York, NY); video recording allowed for retrospective analysis of insect behaviour. The temperature in the arena was recorded by inserting a thermocouple, attached to an electrical thermometer (DTM-315, Tecpel, Taiwan), through the side wall of the arena. Video recording software (Studio Capture DT, Studio86designs, U.K.) allowed arena temperature and time elapsed to be displayed on the video recordings.

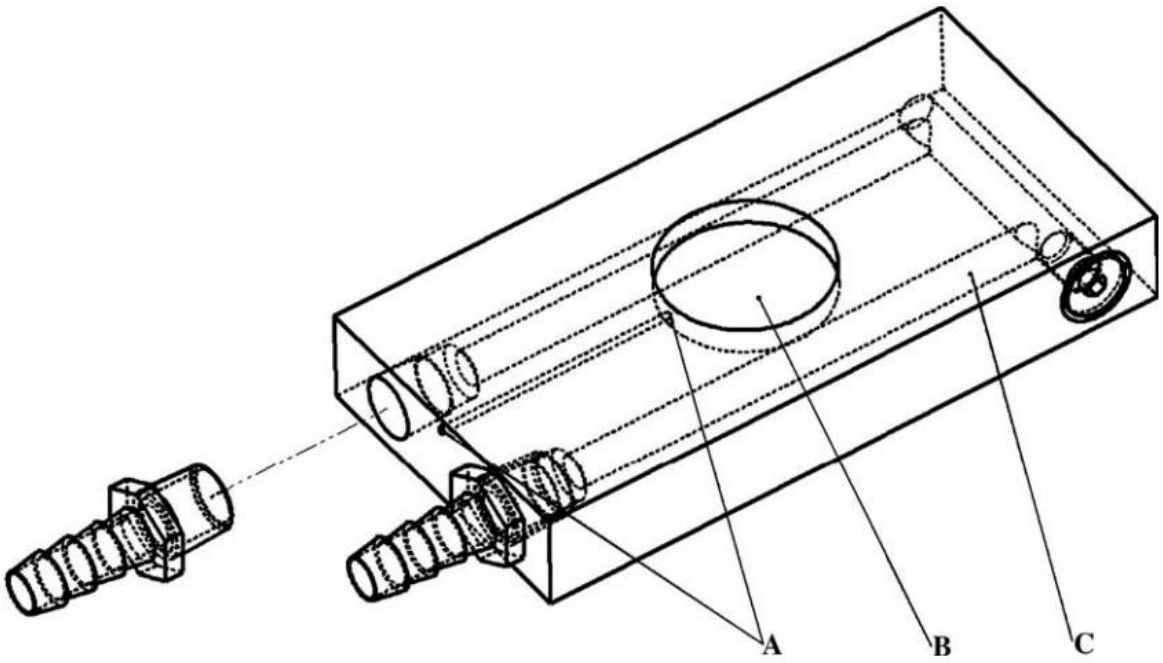

Figure 7-1: Design of the aluminium block system, showing: A) passage for the thermocouple; B) the insect arena; C) channels bored though the block to allow heated or cooled fluid to be pumped around the block (from Hazell *et al.*, 2008).

For both *L. bryoniae* and *D. isaea* it is possible to identify the gender of an individual by eye. Female *L. bryoniae* have a pointed black protrusion from the rear of the abdomen which the

males lack (Minkenberg & Van Lenteren, 1986), whilst male *D. isaea* have a white stripe on the tibia of the hind legs which is absent in the females (Askew, 1968). As gender was easily identifiable in these species males and females have been examined separately, with a sample size of 30 individuals for each gender.

7.3.3. Effect of size on *D. isaea* thermal activity thresholds

The thermal activity thresholds of a second set of thirty male and thirty female *D. isaea* were assessed and a JPEG image was taken of each individual from which the total length of the insect's body (head, abdomen and thorax but not antenna or wings) was measured using ImageJ (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Insect body length was converted into millimetres using the diameter of the arena (25mm) as a calibrated known distance.

7.3.4. Calibration of Results

In the arena system used it became apparent that despite the relatively slow rate of temperature change (0.2°C min^{-1}), a lag existed between the air temperature in the arena and the body temperature of the insects (pers. com. Jiranan Piyaphongkul, however, see Stevenson (1985) for an explanation). For this reason it has been necessary to calibrate insect temperature against arena temperature. In order to do this, five males and five females of each species were euthanaised by freezing. Once the dead insects had equilibrated with ambient temperature in the culturing environment, each insect was attached to a thermocouple with petroleum jelly and placed into the arena, and the ramping procedure described above was followed. Between the highest CTmin and lowest CCT or lowest CTmax and highest HCT the insect body temperature was recorded after every 0.5°C

change in arena temperature. Insect body temperature was then regressed against arena temperature (see Figure 7-2 & Figure 7-3). These regression equations were then used to determine the CTmin/max and chill/heat coma values accordingly. Statistical analysis was performed on these calibrated data.

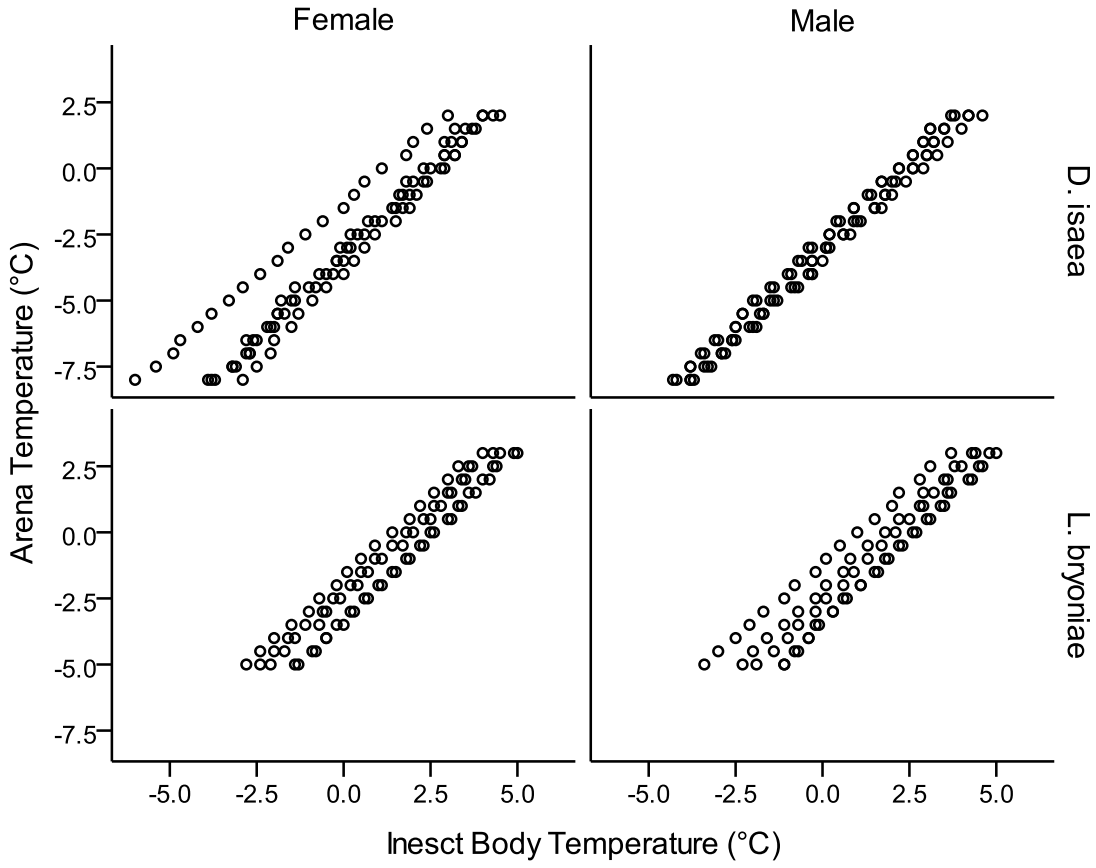

Figure 7-2: Regressions of arena temperature against insect body temperature for the calibration of lower thermal thresholds. For male *D. isaea* (insect body temperature = 2.43 + 0.791 x arena temperature) and female *D. isaea* (insect body temperature = 2.28 + 0.785 x arena temperature) whilst for male *L. bryoniae* (insect body temperature = 2.03 + 0.803 x arena temperature) and female *L. bryoniae* (insect body temperature = 2.05 + 0.799 x arena temperature), with five replicates per treatment.

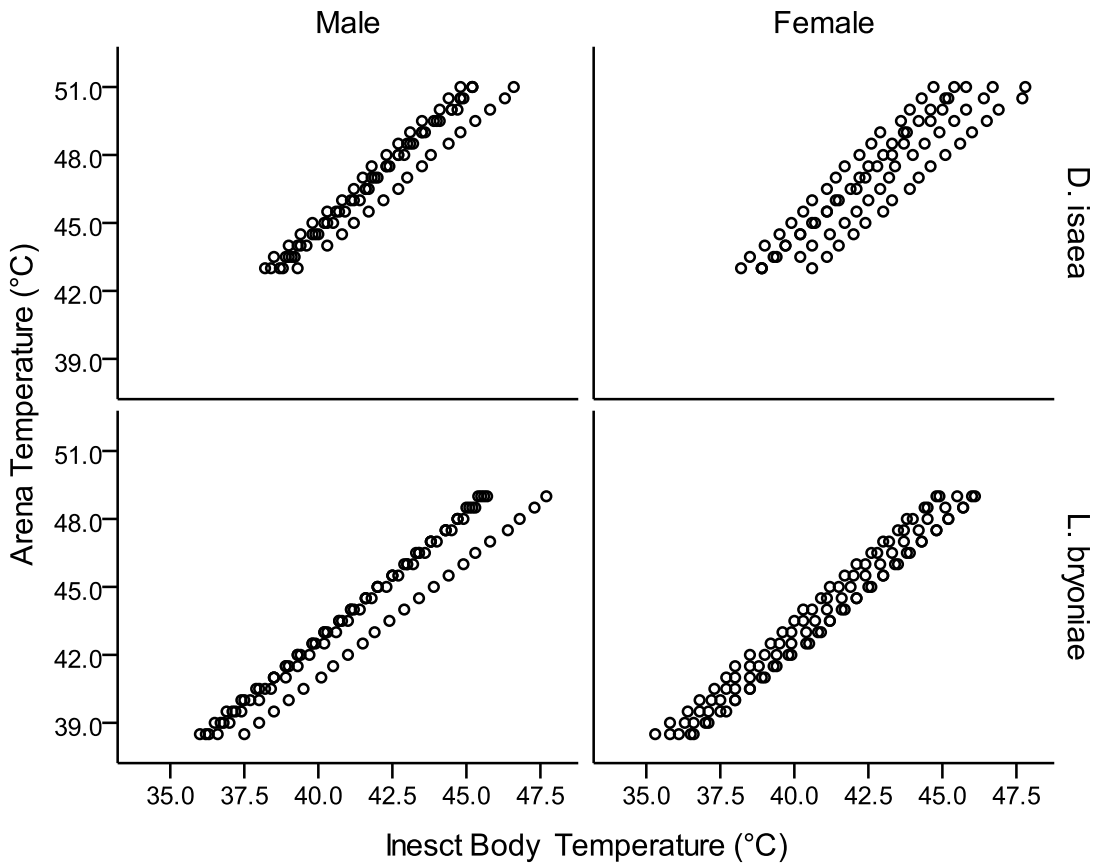

Figure 7-3: Regressions of arena temperature against insect body temperature for the calibration of upper thermal thresholds. For male *D. isaea* (insect body temperature = 2.24 + 0.847 x arena temperature) and female *D. isaea* (insect body temperature = 2.57 + 0.853 x arena temperature) whilst for male *L. bryoniae* (insect body temperature = 1.79 + 0.904 x arena temperature) and female *L. bryoniae* (insect body temperature = 2.00 + 0.888 x arena temperature), with five replicates per treatment.

7.3.5. Statistical analysis

All data was assessed for normality using a Kolmogorov-Smirnov Test; the data for all thermal activity thresholds of each species was normally distributed except for CCT in *D. isaea*. It was not possible through transformation to make this data normal; therefore in

order for comparisons between analyses to be valid, all analysis has been conducted using non-parametric tests. For comparison of thermal activity thresholds between species and within species between genders Mann-Whitney U tests have been utilised.

7.4. Results

L. bryoniae had a median CTmin of 2.4°C and a median CCT of -0.7°C, whilst for *D. isaea* the median CTmin was 0.2°C and the median CCT was -1.8°C (Figure 7-4). In *L. bryoniae*, median upper thermal activity thresholds were CTmax, 41.3°C, and HCT, 43.3°C, and for *D. isaea* CTmax, 42.0°C and HCT, 42.7°C (Figure 7-5) Thus, whilst, *L. bryoniae* is capable of coordinated movement over a range of 38.9°C, from the CTmin to CTmax, *D. iseae* has a greater range of 41.8°C.

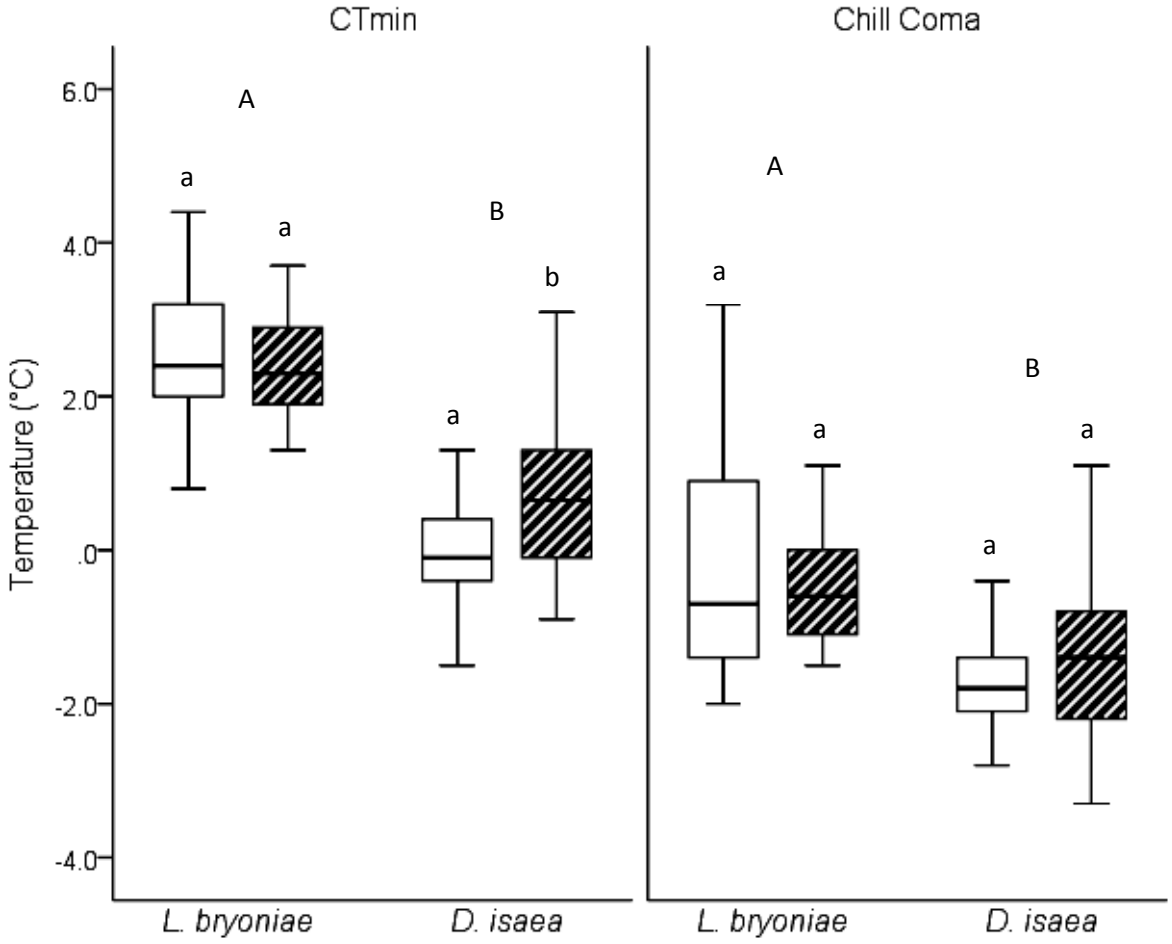

Figure 7-4: Lower thermal activity thresholds (CTmin and CCT), with 95% confidence intervals (clusters defined by sex with females white and males hashed), with thirty replicates of each gender for both species at each threshold. Medians with the same letter (upper case for between species and lower case for between genders within species) are not significantly different at P<0.05.

Comparison of thermal activity thresholds between species revealed significant differences between *L. bryoniae* and *D. isaea* for CTmin ($U_{(120)}$=240.500, Z=-8.191, p<0.001), CCT ($U_{(120)}$=653.000, Z=-6.027, p<0.001), CTmax $U_{(120)}$=1246.500, Z=-2.907, p=0.004) and HCT ($U_{(120)}$=1309.500, Z=-2.577, p<0.010). In all cases the HCT was synonymous with the upper

lethal temperature (i.e. there was no recovery from HCT); however, recovery from CCT was observed in both species.

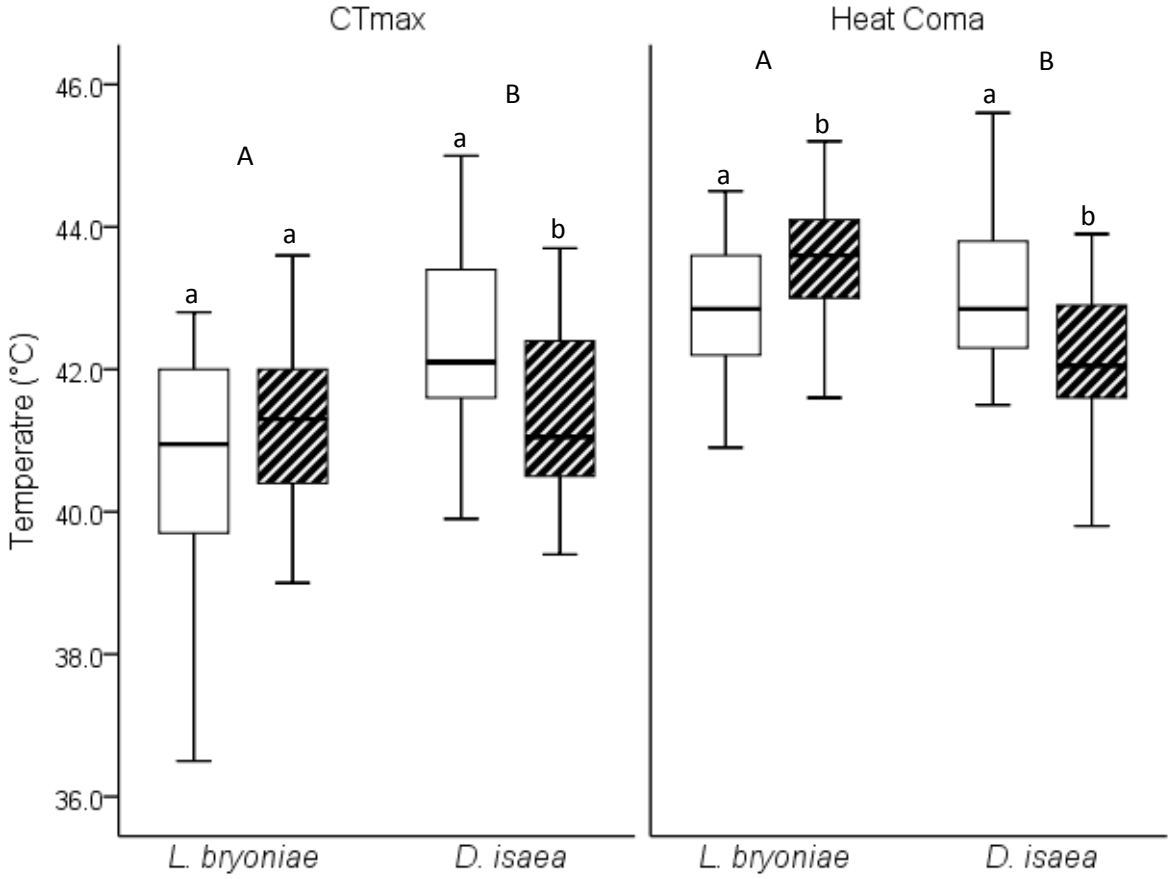

Figure 7-5: Upper thermal activity thresholds (CTmax and HCT), with 95% confidence intervals (clusters defined by sex with females white and males hashed) with thirty replicates of each gender for both species at each threshold. Medians with the same letter (upper case for between species and lower case for between genders within species) are not significantly different at $P<0.05$.

Within species comparisons between males and females revealed that for *L. bryoniae* only the HCT differed significantly between the sexes ($U_{(60)}=295.000$, $Z=-2.294$, $p=0.022$); all other

thermal activity thresholds examined shown no significant difference between the sexes. Thermal activity thresholds in *D. isaea* showed more differences between the sexes with CTmin ($U_{(60)}$=231.500, Z=-3.236, p=0.001), CTmax ($U_{(60)}$=249.000, Z=-2.974, p=0.003) and HCT ($U_{(60)}$=229.500, Z=-3.265, p=0.001) all showing significant differences, but with no difference in CCT between male and female *D. isaea*.

7.4.1. Effect of size on *D. isaea* thermal activity thresholds

The median body length of male *D. isaea* assessed for lower thermal activity thresholds was 1.24mm; female *D. isaea* with a median length of 1.54mm are significantly larger ($U_{(60)}$=136.500, Z=-4.635, p<0.001). The median body length of *D.isaea* used for upper thermal thresholds was 1.14mm and 1.26mm for males and females respectively with females again being significantly larger ($U_{(60)}$=317.500, Z=-1.959, p=0.05). Correlations between size and lower thermal threshold temperatures (Figure 7-6) indicated a significant negative association for both CTmin (r_s=-0.602, d.f.=58, p<0.001) and CCT (r_s=-0.260, d.f.=58, p=0.045); however, when the genders are examined separately, no significant association was found between body length and CTmin or CCT in female *D. isaea* or CCT in male *D. isaea*, the exception being CTmin in males which shows a strong negative association with body length (r_s=-0.619, d.f.=28, p<0.001). No significant associations were found between *D. isaea* body length and upper thermal activity thresholds (Figure 7-7) for combined or separate genders. Comparison of thermal activity thresholds between genders revealed significant differences for CTmin ($U_{(60)}$=202.000, Z=-3.668, p<0.001), CCT ($U_{(60)}$=283.000, Z=-2.470, p=0.014), CTmax ($U_{(60)}$=248.000, Z=-2.987, p=0.003) and HCT ($U_{(60)}$=221.000, Z=-3.388, p=0.001).

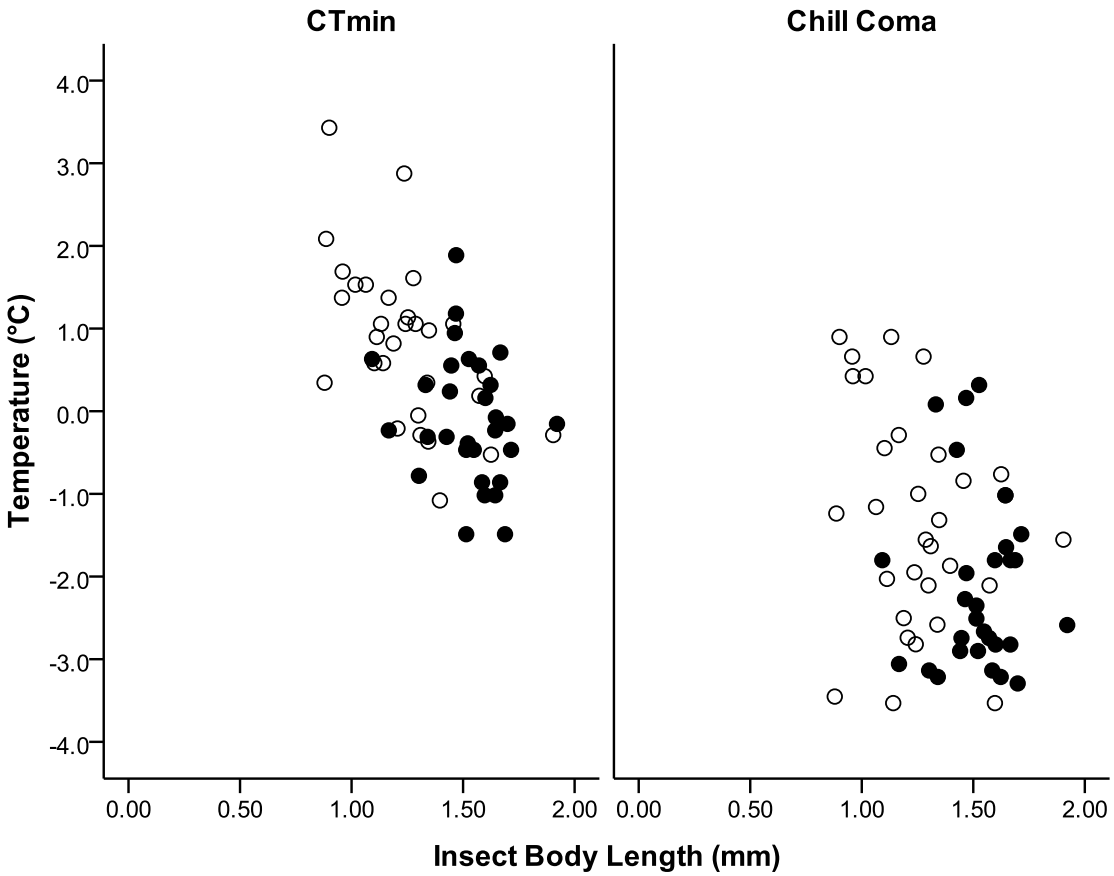

Figure 7-6: Individual lower thermal tolerances for male (white) and female (black) *D. isaea* in relation to body length (mm), with thirty replicates per gender for each treatment. For total populations (males and females) both CTmin (r_s=-0.602, d.f.=58, p<0.001) and CCT (r_s=-0.260, d.f.=58, p=0.045) show significant negative associations with insect body length.

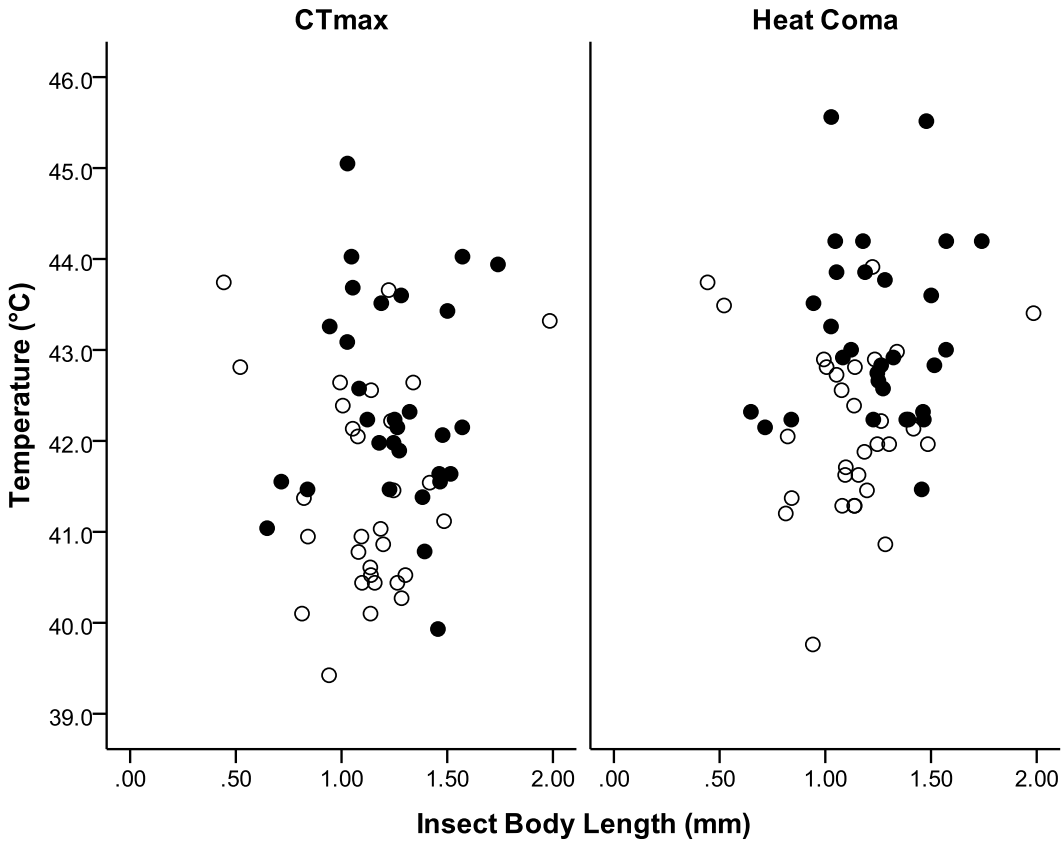

Figure 7-7: Individual upper thermal tolerances for male (white) and female (black) *D. isaea* in relation to body length (mm), with thirty replicates per gender for each treatment. At $P<0.05$ no significant associations were found between CTmax or HCT with insect body length.

7.5. Discussion

It is generally assumed that smaller insects which have a higher surface-area to volume ratio are more resistant to low temperatures (Le Lann *et al.*, 2011; Renault *et al.*, 2003; Storey & Storey, 1992) whilst larger insects with a lower surface-area to volume ratio will be more desiccation resistant (Block, 1996; Duncan & Lighton, 1994) and therefore more heat resistant (Le Lann *et al.*, 2011). The results presented in the current study only partially

reflect this, as the smaller species (*D. isaea*) has showed greater resistance to low temperatures than the larger *L. bryoniae*, having lower CTmin and CCT values (Figure 7-4). However, the difference in CTmax was not as would be expected, with *D. isaea* again having greater resistance despite being smaller (Figure 7-5). This result is especially surprising as the darker colour of *D. isaea* would also be associated with greater radiative heating (Trullas, van Wyk, & Spotila, 2007).

Although the observations made in the current study were symptomatic of neuromuscular transmission failure at extreme temperatures (Hosler, Burns, & Esch, 2000; Klose & Robertson, 2004), one of the potential risks to an insect of exposure to high temperature is dehydration or even desiccation. Le Lagadec *et al.*, (1998) have shown that larger body size increased resistance to desiccation in seven species of keratin beetle; however, size and water loss is not a simple relationship, with factors such as cuticular permeability (Schilman, Lighton, & Holway, 2005; Willmer, 1980), waterproofing by epicuticular lipids (Hood & Tschinkel, 1990) and water content (Duncan & Lighton, 1994) also affecting water loss and desiccation tolerance. The results show that *D. isaea* maintained coordinated movement at higher temperatures than *L. bryoniae* and differences in desiccation resistance or tolerance may partially explain this difference.

For both species heat coma was observed to be synonymous with death as no individuals showed any signs of recovery, once removed from the arena. This responce has been observed in other insects assessed using the same method, including brown plant hopper (Piyaphongkul, Pritchard, & Bale, 2012) and the peach-potato aphid (Hazell *et al.*, 2008).

Within species there was a surprising lack of difference between male and female *L. bryoniae* despite the size difference, with males typically being smaller, although individual *L. bryoniae* were not measured in the current study. Further to which the difference observed between male and female *L. bryoniae* in HCT was not as would be expected (Figure 7-5), with males showing greater heat resistance despite being smaller. Similarly, the gender-specific pattern of thermal tolerance in *D. isaea* cannot be explained simply in terms of size, as *D. isaea* females were always more resistant despite being larger, which is as would be predicted for HCT and CTmax but not CTmin or CCT. However, haplo-diploid sex determination in parasitoids (Roux *et al.*, 2010) and gender specific differences in HSPs (Chown & Nicolson, 2004; Folk *et al.*, 2006; Goto & Kimura, 1998) have been reported as influencing gender-specific patterns of thermal tolerance.

Diglyphus isaea were individually measured; however, when genders were analysed separately no association between thermal tolerance and body length was found in females, and only with CTmin in males (Figure 7-6). The size-thermal tolerance relationship, postulated by Le Lann *et al.*, (2011), looks weak, given so many exceptions to the rule suggesting that when size differences are measured in fractions of a millimetre other factors e.g. between species or genders differences, phenotype/genetic differences and reflectivity, may be of greater importance.

From a biological control perspective it is advantageous for a control agent to remain active at temperatures above and below those which immobilise their prey or hosts (Hughes *et al.*, 2010). In general, *D. isaea* was more resistant to extremes of temperatre than *L. bryoniae*, the exception being that the HCT of *L. bryoniae* was greater than that of *D. isaea*.

Importantly however, *D. isaea* maintained coordinated movement at temperatures both below and above those at which *L. bryoniae* attained its CTmin or CTmax respectively. Further work, however, on the temperature dependent efficacy of *D. isaea* as a biological control agent of *L. bryoniae* could examine the thermal limits of fertility and the threshold temperatures for flight in both species.

8. GENERAL DISCUSSION

Biological Control has a number of advantages over the use of conventional chemical pesticides, not least among these is the compatibility of biological control with the use of insect pollinators (Blockmans, 1999). In crops such as tomato which require buzz-pollination the use of bees as opposed to manual pollination may be more cost efficient for growers (Dogterom, Matteoni, & Plowright, 1998). Another advantage is the much reduced possibility that a pest will develop resistance (Bale *et al.*, 2008; Blockmans, 1999; van Lenteren, 2000), an issue which has occurred repeatedly in the conventional control of leaf miners, since the resistance of *Liriomyza spp.* to DDT was demonstrated in the 1950s (Ferguson, 2004; Hills & Taylor, 1951). Despite this fact, the efficacy of *Diglyphus isaea* as a control agent of *Liriomyza bryoniae* is anecdotally reported to have waned over the past decade.

Liriomyza bryoniae is a polyphagous leaf mining fly and pest of many vegetable and ornamental crops in European glasshouse agriculture, damaging plants through leaf mining and stippling (Minkenberg & van Lenteren, 1986; Parrella *et al.*, 1985). The native European parasitoid *D. isaea* has been commercially available as a biological control agent for use against *L. bryoniae* and other leaf mining dipterans since 1984 (Malais & Ravensberg, 2003). Control of leaf miners by *D. isaea* was initially thought to be excellent (Benuzzi & Raboni, 1992 cited in Nedstam & Johansson-Kron, 1999); however, recent anecdotal reports from tomato growers suggest that the level of control provided by *D. isaea* has declined, although, contrary reports have been made regarding the level of control in other crops.

This project first examined the different levels of control in three crops under standard conditions and then examined possible factors that may affect levels of control. Specific issues addressed were: the role habituation of the pest and/or parasitoid to the host plant may play, the role of the natal host-complex on the searching behaviour of *D. isaea*, the effect of different ontogenetic stages of plant development on the pest and parasitoid, and the relative critical thermal limits of the pest and parasitoid.

8.1. Differences in *Diglyphus isaea* efficacy between crops

The ability of conventionally-reared *D. isaea* to utilise *L. bryoniae* as a host in three crops, French bean, gerbera and tomato, was examined. The levels of *L. bryoniae* infestation varied greatly between the three plants, with no mines forming in gerbera. Between French bean and tomato there were significant differences in the numbers of *L. bryoniae*, with the greater number being supported by French bean for every life stage examined (Figure 3-8). There was also a marked difference in larval mortality, with just 37% of larvae from tomato plants successfully forming pupae compared with 96% of *L. bryoniae* larvae in French bean. Consequently, *D. isaea* could not initially be reared from the tomato-*L. bryoniae* host complex (sections 3.4.1 & 3.4.2). Subsequently, however, *D. isaea* has been reared from *L. bryoniae* in tomato (sections 3.4.3 & 3.4.4) with this host-complex producing a similar number of *D. isaea* to the French bean-*L. bryoniae* host complex. Similarly, the pupae of *L. bryoniae* that had been reared on tomato were found to be significantly smaller than those which had been reared from French bean (Figure 3-14). However there was no difference in the length of *D. isaea* wings, regardless of the *L. bryoniae*-plant host combination they were reared from (Figure 3-16).

Although the differences in terms of *D. isaea* production did not vary significantly between the culturing host French bean and target crop tomato, at best, a mean of 78.25 *L. bryoniae* larvae from tomato gave rise to just 5.25 *D. isaea* offspring per female, suggesting that concerns about the efficacy of *D. isaea* as a biological control agent of leaf miners in this crop may have some foundation.

8.2. To persist with *Diglyphus isaea* or to find an alternative?

Several factors suggest that *D. isaea* is, or at least has the potential to be, an effective control agent against *L. bryoniae*. Firstly, *D. isaea* is able to utilise *L. bryoniae* larvae in tomato plants as a host (sections 3.4.3 & 3.4.4), the habituation of *L. bryoniae* to tomato plants, despite smaller leaf miner larvae (Figure 4-4), does not affect the ability of *D. isaea* to utilise them as hosts (Figure 4-5), nor does it affect the size or sex ratios of *D. isaea* reared on tomato-habituated *L. bryoniae* (Figures 4-6 & 4-7). The natal host complex of *D. isaea* has not shown any effect in terms of the number of offspring produced, nor does it affect the size or sex ratios of the offspring (Figures 4-9 & 4-10).

Secondly, it is desirable that a biological control agent should remain active at temperatures beyond those which cause their target to become immobilised (Hughes *et al.*, 2010). Thermal data collected in the current study suggests that *D. isaea* has this capacity to be an effective control agent of *L. bryoniae*, both early in the season when temperatures are still low and during peak summer temperatures. *Diglyphus isaea* maintains coordinated movement at temperatures as low as 0.2°C, whereas *L. bryoniae* adults become uncoordinated at 2.4°C, also *D. isaea* also has a higher CTmax than *L. bryoniae*, with the upper limits of coordinated movement being 42.0°C and 41.3°C respectively. Whilst these

results can be viewed as positive in terms of the efficacy of *D. isaea* as a biological control agent of *L. bryoniae*, it was not as predicted by hypotheses linking insect size to thermal tolerance (Le Lann *et al.*, 2011), which suggest that as *D. isaea* is smaller than *L. bryoniae* it would be expected to succumb more easily to high temperatures than would the larger host species.

Finally, *D. isaea* shows no signs of imprinting on its natal host, responding equally to the odours of *L. bryoniae*-infested French bean and *L. bryoniae*-infested tomato leaf segments in Y-tube olfactometer tests (Figure 6-2). Therefore, it would be tempting to think that concerns regarding the efficacy of *D. isaea* mass reared on *Liriomyza spp.* in French bean but targeted against *L. bryoniae* in tomato may be unfounded. However, *D. isaea* does not appear to utilise herbivore induced plant volatiles as a host location cue, at least not under the conditions of the Y-tube or Trap assay experiments. Nearly one third of the *D. isaea* tested in the Y-tube olfactometer failed to display any preference for either odour cue, whilst, *D. isaea* females also failed to show any preference between *L. bryoniae*-infested and uninfested French bean leaf segments in Trap assay experiments (Section 6.3.2) over it is difficult to draw any meaningful conclusions from these data.

There are also a number of other indicators which may raise concern about the future use *D. isaea* as a control agent against *L. bryoniae*. Firstly, whilst tomato-reared *D. isaea* produced offspring in equal numbers to French bean-reared *D. isaea* in tomato, they did not appear to have as higher kill rate, with more *L. bryoniae* larvae surviving to pupation in the presence of tomato-reared *D. isaea* than in the presence French bean-reared *D. isaea* (Figure 4.8). Secondly, whilst *D. isaea* did utilise *L. bryoniae* hosts in juvenile plants, the presence of *D.*

isaea had no significant effect on the number of *L. bryoniae* larvae surviving to adulthood (Figure 5.4), suggesting that the release rate of three *D. isaea* females to a mean of 78 *L. bryoniae* larvae was too low and that a higher release rate would be needed for effective control. Finally, in the same study *D. isaea* were apparently unable to utilise *L. bryoniae* larvae in mature plants (Figure 5-7), which is of major concern, as *D. isaea* is generally used in the warmer months when tomato plants will be mature with *Dacnusa sibirica* being used earlier in the season (Malais & Ravensberg, 2003).

Despite the loss of confidence by growers, biological control companies may choose to persist with *D. isaea* rather than develop alternative biological control agents, as *D. isaea* is native to Europe (Minkenberg, 1989) whereas other parasitoids which show potential as biological control agents of *Liriomyza* leaf miners originate from the new world. Also it would appear that the most promising agent *Diglyphus begini*, whilst effective against those *Liriomyza* species introduced from the new world, *L. trifolii* and *L. huidobrensis*, is not able to utilise *L. bryoniae* as a host at least not under laboratory conditions (pers. comm. J. Klapwijk). The use native natural enemies against *L. bryoniae* is not subject to the regulations in many countries that might affect the introduction of a non-native natural enemy (Bale *et al.*, 2008). Further to which, unlike in North America where a coordinated cross-border system of regulation exists, in Europe, there is not yet a single regulatory body (Hunt *et al.*, 2008) and many separate licences may have to be applied for in different countries thus escalating the cost of bringing a new biological control agent onto the European market (Bigler *et al.*, 2005).

8.3. Future work

It seems unlikely that in the short term at least companies producing *D. isaea* as a biological control agent will choose to discontinue its use and search for new agents to utilise in the control of *L. bryoniae*. However, it is not clear whether the previously apparently successful control of *Liriomyza* species by *D. isaea* could be attributed to this species, or to *D. isaea* alone; and for the same reason, there must be doubt that there has been actually been a decline in its efficacy over the past decade. Never the less, there are a number of other aspects of this complex problem which as yet have not been addressed. Leading directly on from the current study, the Y-tube olfactometery and Trap assay work could be repeated using whole plants as the cocktail of volatiles is likely to different between whole plants and small segments (Wei *et al.*, 2006). With regard to the thermal ecology of *D. isaea* and *L. bryoniae*, examination of thermal limits to reproduction and threshold temperatures for flight may improve our understanding of how the abiotic environment interacts with these two species. Other work on the efficacy of *D. isaea* not directly related to the current study might include an examination of the possibility of cryptic species in both *L. bryoniae* and *D. isaea*, using the approaches of Scheffer and Lewis (2006), and Sha *et al.* (2007). Finally, studies with wild caught insects from across their European ranges would enhance our understanding of what is taking place in the glasshouses of southern Europe where control appears to be satisfactory compared with northern Europe were the reported problems have occurred.

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