# PREDATOR-PREY BEHAVIOURAL INTERACTIONS ON PLANTS, WITH SPECIAL EMPHASIS ON APHID DROPPING DEFENCE AND LADYBIRD SEARCH STRATEGIES

Rosalind Kay Humphreys

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# Predator-prey behavioural interactions on plants, with special emphasis on aphid dropping defence and ladybird search strategies

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St Andrews

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# Abstract

Predator-prey interactions have significant short- and long-term implications for the individuals involved, their populations' dynamics, and the broader ecological community. Predation is a strong selective force that has led to prey evolving a great diversity of defensive adaptations, while predators undergo strong selection to successfully locate and capture prey. For both 'sides' of these interactions, behavioural adaptations offer flexibility in deployment, and so are expected to be context-sensitive. In this thesis, I use the ladybird-aphid predator-prey system to examine both prey defence behaviours and predator search strategies. Considering the prey, dropping – a widespread but understudied antipredator behaviour – is my focus. My findings suggest that, although dropping seems superficially simple, the selective pressures on it are complex. Dropping is affected by internal factors, such as genotype and the presence of endosymbionts. The nature and proximity of the predatory threat also affect dropping. Further, the consequences of dropping depend on many factors, including where on a plant predator-prey encounters occur; this in turn will be influenced by predator search strategies. Considering predator search, my focus is ladybird movements when searching branched environments, particularly potential turning biases and turn alternation. My work suggests that predators do not follow simple rules when searching a plant for prey, but they may exhibit some innate turning tendencies under particular conditions. Predator search strategies will be highly influential in affecting the context (e.g. location on plant) in which predator-prey interactions take place. Future work should investigate predator search further in order to provide a deeper understanding of the selective pressures on dropping in prey. For both prey dropping and predator search strategies, researchers must also appreciate the context-sensitivity of the different behaviours, and work to understand influencing cues, triggers, and outcomes specific to the species and circumstances involved.

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# Chapter 1: General Introduction

For at least part of their lives, most animals are predators, prey, or both. As such, the interactions between predators and their prey – whether these are defined by narrow or broad spatiotemporal scales – firstly, often play a central role in the fitness of any given individual. Secondly, they are important determinants for the relative success and dynamics of both predator and prey populations. And, thirdly, they carry significant consequences for the overall structure and function of broader ecological communities (Barbosa and Castellanos, 2005). Over evolutionary time, an 'arms race' of adaptations and counter-adaptations can occur between predators and prey: with natural selection acting to increase prey's effectiveness at avoiding detection and escaping predators, while concurrently acting to increase the efficiency with which predators search for and capture their prey (Davies and Krebs, 2009). The resulting adaptations on both 'sides' of the arms-race, and the interplay between them, determines the outcome of encounters between predators and their prey. Although this thesis focuses on particular prey and predator species, many of the themes covered are relevant to predator-prey interactions more broadly, and similar behavioural adaptations to the ones discussed are seen across the animal kingdom.

The threat of predation is a major selective force for prey species. As a consequence of their long coexistence with predators, and the fact that most prey species are exploited by multiple predatory species, prey typically possess a portfolio of defensive adaptations (Caro, 2005, Ruxton et al., 2018, Sugiura, 2020). These antipredator adaptations, be they morphological, physiological, or behavioural, increase the likelihood of survival at various stages of the predation sequence (Endler, 1991, Ruxton et al., 2018). However, behavioural defences usually offer more flexibility in their deployment than other types, often allowing for more context-sensitive responses to specific predators or to the prey's current internal state and/or environmental conditions. Dropping is one such antipredator behaviour that can be used flexibly by prey. As I set out in more detail in Chapter Three, I define dropping as a voluntary antipredator defence whereby a prey individual uses gravity, wind or water currents to power escape from imminent threat. Despite the fact that dropping is a common and taxonomically widespread defence, it is understudied and underappreciated as a significant component of many predator-prey interactions. Contributing to knowledge on antipredator dropping behaviour is, therefore, a major aim of this thesis.

The biological and ecological adaptations that facilitate predators finding their prey also include important behavioural adaptations and decision-making when searching environments. The effectiveness of any method by which a predator searches its environment will depend on the availability of its prey and their spatial and temporal distribution. But, given that a predator's searching behaviour will at least partly have a genetic basis, over evolutionary time, natural selection can be expected to hone - to some extent - predators' abilities to efficiently locate their prey (Bell, 2012). How a predator explores its environment can also be expected to have a significant bearing on how effective different antipredator defences may be in mitigating the predation risk experienced by prey. Although the search strategy of predators clearly constitutes a fundamental aspect of predator-prey interactions, establishing the existence of innate tendencies or stimulus-sensitive behaviours that have evolved to enable efficient prey-finding is often difficult because of the practical challenges involved in tracking predator behaviours and environmental conditions over long stretches of time and space, through complex habitats. Even where the habitats in, or structures on, which predators search are relatively small in scale, developing a clear understanding of search strategies can be hindered by a lack of knowledge regarding a predator's cognition and sensory abilities. Another primary aim of this thesis, therefore, is to test and generate some hypotheses concerning predator search strategies that lead to encounters with prey.

The predator-prey interaction this thesis focuses on is that between ladybird beetles (family Coccinellidae) and aphids (family Aphididae). This study system was chosen in part because of the significance of aphids as economically important pests of agricultural crops (van Emden and Harrington, 2007, Sullivan, 2008, Dedryver et al., 2010) and ladybirds as some of their major natural enemies with the potential to be managed as effective biological control agents (Hodek and Honek, 1996, Obrycki and Kring, 1998, Dixon, 2000). Aphids, particularly pea aphids (*Acyrthosiphon pisum*), are also the taxa in which antipredator dropping behaviour has received the most attention so far; this is likely partly due to the relative ease of studying such small and rapidly reproducing prey species in which the defence occurs. Nonetheless, there remains much to learn about the triggers, trade-offs, and consequences of dropping in aphids, especially in species beyond pea aphids. Considering the foraging of predatory ladybirds, many aspects of their behaviours and sensory abilities are not well understood, even in the most well-studied species (Hodek and Michaud, 2008, Norkute et al., 2020). Further, most studies testing theoretical foraging strategies and use of cues by ladybirds have taken place under laboratory conditions and/or involve observations of insect movements on artificial rather than natural materials (Pervez and Yadav, 2018); there is therefore a need for studies utilising more naturalistic conditions in order to develop a fuller picture of ladybird search tendencies on plants in the wild. For further details of the ladybird-aphid predator-prey system and focal study species in this thesis, see Chapter Two.

In order to address both ‘sides’ of predator-prey behavioural interactions on plants, this thesis is largely structured in two halves, the first addressing aphid antipredator responses with a focus on dropping. Dropping receives particular attention as a response to the threat of predation in this thesis because of its current understudied status. In Chapter Three, I review dropping as an antipredator defence, highlighting key findings concerning the use of dropping by aphids and identifying important gaps in the scientific literature. Chapters Four, Five and Six then report a series of findings resulting from experimental work conducted at the James Hutton Institute (JHI) in Dundee, wherein the antipredator responses of pea aphids and potato aphids (*Macrosiphum euphorbiae*) to foraging predators on live plants were examined. Chapter Four describes the methodology for this work in-depth, and explores the existing suggestion that facultative endosymbionts harboured by aphids can influence their defensive behaviours. Infection with the *γ*-proteobacterium *Hamiltonella defensa* has been reported to confer resistance against parasitoids to pea aphids, but may also reduce their use of defensive behaviours against predators (Oliver et al., 2003, Dion et al., 2011, Polin et al., 2014). Chapter Four tests whether infection with the symbiont *Hamiltonella defensa* affects the antipredator responses of pea aphids to different predator types, and whether symbiont infection and/or genetic parasitism resistance affects the antipredator responses of potato aphids to different predator types. In this chapter, some antipredator defences are grouped in the analysis in order to keep focus on the overall influence of the internal and external factors being examined. Chapter Five then examines more closely the specific use of antipredator dropping behaviour in these two aphid species relative to a range of alternative behaviours, and its timing in relation to contact with predators, when faced with the different predator types: ladybird (*Adalia bipunctata*) adults and lacewing (*Chrysoperla carnea*) larvae. Chapter Six then turns attention to one of the most neglected aspects of dropping behaviour – what happens to individual prey after they have dropped. This chapter focuses on potato aphids, for which more post-dropping data was collected during my experiments, and explores the potential influence of a range of ecological factors on aphids’ post-drop durations of tonic immobility, recovery rates, and occurrences of ‘instant recovery’ (where dropped aphids re-cling to lower plant parts rather than falling to the underlying substrate).

Chapter Seven acts as a linking chapter between the two halves of the thesis, describing an exploratory field study that investigated both antipredator dropping by wild aphids and ladybird searching behaviour in a natural context. Rather than test specific research questions, its purpose was to collect exploratory data on both of these components of predator-prey interactions - using aphids sourced opportunistically on a variety of wild, living plants - and to generate ideas and hypotheses to motivate and direct future research. The next two chapters then constitute the second half of the thesis, investigating the potential existence of two behavioural tendencies that could determine, to some extent, ladybird searching behaviour on plants. Chapter Eight describes a study designed to test a previous study’s finding that ladybirds display significant, innate turning biases (or ‘handedness’); a strategy that would, theoretically, improve foraging efficiency on branching structures (Girling et al., 2007); under different conditions. Finally, Chapter Nine describes two studies designed to examine consecutive turning choices and the possibility of an innate preference for turn alternation influencing ladybird movements when navigating branching structures. As far as I am aware, these experiments constitute the first tests for such a tendency in ladybirds. While the first study focussed on the relative frequencies of turn alternation and turn repetition, the second study factored in a range of environmental variables that might contribute to the turning decisions made by ladybirds, including branch thickness and the difference of the angles presented by each branching option compared to the present heading or compared to the direction of gravity. Chapter Ten offers a general discussion of emerging themes from my results across the thesis, and offers a perspective on how my studies might most effectively be developed further.

In the next short chapter I provide a brief overview of aspects of the biology of the study species that have a bearing on the work presented in this thesis.

# Chapter 2: Basic biology

The interactions between aphid prey and their ladybird predators are the focus of this thesis. Below, the basic biology of these taxa are outlined, but see the Appendix for more details.

## 2.1 Aphids (Aphidoidea)

Aphids are small (1-10mm long), soft-bodied insects belonging to the superfamily Aphidoidea within the order Homoptera, the plant-sucking bugs (Dixon, 2012). They have a worldwide distribution, though species are more abundant in temperate latitudes compared to the tropics (Sullivan, 2008). Diagnostic features of their morphology include: a proboscis situated between the forelegs, a pair of siphunculi (also known as cornicles) that can release a sticky fluid when the aphid is disturbed, a tail-like cauda, and antennae (Sullivan, 2008, Dixon, 2012). 'Apterous' forms are wingless, but 'alate' forms additionally possess wings (Sullivan, 2008). Aphids also possess a variety of attachment mechanisms associated with their feet ('tarsi') that help them to grip plant surfaces (Kennedy, 1986, Beutel and Gorb, 2001, Dixon, 2012).

Aphid species can exhibit a wide variety of body colours, spanning from almost colourless to pale yellow, green, brown, black, or even pink (Tsuchida, 2016). Often their colouration is cryptic against host plant structures (Dixon, 1973, Dixon, 2012), a feature that might have evolved in part to reduce predation (Tsuchida, 2016).

The life histories of aphids show great diversity, with the complexity and variability of their annual cycle varying both between and within species (Hales et al., 1997). Species for which parthenogenetic reproduction is interrupted by annual sexual reproduction are known as 'holocyclic' (Llewellyn, 1984, Sullivan, 2008). Whether aphids are born live from viviparous parthenogenetic females or hatch from an egg following mating, they follow the same developmental sequence: usually four instars or nymphal stages, then adult (Sullivan, 2008). For 'heteroecious' aphid taxa, an alternation of host plants is an obligate part of their annual cycle (Hales et al., 1997), whereas 'monecious' species remain on one host species throughout the year (Sullivan, 2008). Aphids are either generalist, able to feed on a large number of host plant species or even families, or more host-specific (Hales et al., 1997). Most species of aphids probe needle-like structures known as stylets into plants' internal structures to feed on phloem sap, which is rich in sugars but relatively poor in amino acids (Sullivan, 2008, Dixon, 2012). Aphids are entirely dependent on this diet to acquire nitrogen, and so they need to feed continuously, process large quantities of food, and use the nitrogen it contains effectively (Dixon, 2012). However, as their diet is sugar-rich but poor in amino-nitrogen, excess sugar is ingested to obtain scarce nitrogen. This results in the production of honeydew, a sugary mixture of nitrogenous and faecal excretory material that is secreted from the anus and often sticks to aphids' feeding surfaces (Llewellyn, 1984). Aphids also harbour the obligate bacterial endosymbiont *Buchnera aphidicola,* which supplements aphids' unbalanced diets by synthesising essential amino acids, vitamins, and sterols (Clark et al., 2010, Skaljac, 2016). Aphids can also host a range of facultative symbionts, which are not essential but can variously influence fitness (Clark et al., 2010) – these are discussed further in Chapter Four.

**Figure 2. 1:** Adult pea aphid (*Acyrthosiphon pisum*).

Under suitable environmental conditions aphids can complete their life cycle in a very short time, primarily due to their rapid larval growth, small adult size, and parthenogenetic, viviparous reproduction (Llewellyn, 1984). Population sizes can increase rapidly, which can cause catastrophic damage to the yields of agricultural crops that serve as host plants for aphids (Hales et al., 1997). Damage can be direct, by nutrient drain when feeding, or indirect, by a number of means including transmission of viruses and coating with honeydew, which can act as a medium for moulds or fungi or attract other pest species (Hales et al., 1997, Sullivan, 2008, AHDB, 2015). However, aphids are also important food for predators and hosts for parasitoid wasps (Llewellyn, 1984, Sullivan, 2008). The potential of natural enemies to reduce aphid numbers is an important aspect of pest regulation strategies, but aphid antipredator mechanisms (including behaviours discussed in Chapters Three, Four, Five, Six, and Seven) can mitigate such suppression.

There are over 4000 species of aphid (Sullivan, 2008, Dixon, 2012), but the species primarily examined in this thesis – pea aphid, *Acyrthosiphon pisum* (Harris) and potato aphid, *Macrosiphum euphorbiae* (Thomas), both in the tribe Macrosiphini (Stekolshchikov and Buga, 2020) – are two economically important pests of agricultural crops (Sullivan, 2008). The pea aphid is a large, usually green aphid (though there are red-pink morphs) with a pear-shaped body (see Figure 2.1), and long antennae, legs, and cornicles (Sullivan, 2008, AHDB, 2015). Alate females grow to 2.5-3mm long and have deep red or black eyes and yellowish-green antennae, while apterous females have similar features but with a smooth and shiny skin (AHDB, 2015). Pea aphids are monecious, living on herbaceous plants (including legumes such as alfalfa, clover, peas, and broad beans) throughout the year (AHDB, 2015). Sandhi and Reddy (2020), and references therein, provide a detailed description of the life cycle and biology of pea aphids. The primary predators of pea aphids are ladybirds and syrphid larvae (Ximenez-Embun et al., 2014), but other natural enemies include spiders, parasitoid wasps, and fungal pathogens (AHDB, 2015). Similar to pea aphids, potato aphids are a relatively large, pear-shaped aphid species, with wingless adults reaching 2.5-4mm long (AHDB, 2015). They have long legs, antennae, cornicles, and cauda and, like pea aphids, can also be found as both green and red-pink morphs (Sullivan, 2008). But potato aphids have a characteristic dark longitudinal stripe running down their dorsal surface and winged adults usually have a yellowish-brown head and green thorax (AHDB, 2015). In temperate regions potato aphids are heteroecious, with primary host plants including rose, weeds, and potato sprouts (AHDB, 2015, Xu and Gray, 2020) and secondary host plants including a wide range of crops (AHDB, 2015). Natural enemies of potato aphids include: ladybirds, lacewings, parasitoid wasps, predatory flies, spiders, ground beetles, rove beetles, and insect-pathogenic fungi (AHDB, 2015).

## 2.2 Ladybirds (Coccinellidae)

Ladybirds, also known as Coccinellidae, are an ecologically and morphologically diverse family of flight-capable small beetles from the order Coleoptera (Seago et al., 2011). They are common wherever aphids are found in the world (Sullivan, 2008). Adults range in length from 1mm to over 10mm, have domed bodies, short, club-shaped antennae, morphological mechanisms for surface adhesion, and usually distinctive colourful markings (Dixon, 2000, Sullivan, 2008, Moon et al., 2012, Peisker et al., 2013, AHDB, 2015, Heepe et al., 2016). Like other Coleopterans, adult ladybirds possess tough forewings (known as elytra) which conceal well-developed hind wings used for flight (Kukalová-Peck and Lawrence, 1993), as can be seen in Figure 2.2. The mainly aphidophagous Coccinellinae subfamily are generally patterned with two strongly contrasting colours, while coccidophagous subfamilies are mainly black or brown (Dixon, 2000). Depending on species and context, colouration could serve adaptive functions of: crypsis, signalling for mates, and/or thermoregulation (Sloggett et al., 1998).

**Figure 2. 2:** Adult seven-spot ladybird (*Coccinella septempunctata*): **a)** walking with elytra concealing hind wings, and **b)** pre-flight, with hind wings displayed.

Ladybirds exhibit a broad trophic diversity, encompassing herbivory, pollenophagy, fungivory, and highly specialised predation on aphids and other invertebrates (Seago et al., 2011). Most species of ladybird are generalist and polyphagous, feeding on a rather wide range of prey and foods, while relatively few are monophagous, specialising and successfully developing on only a narrow range (Hagen, 1962, Hodek and Evans, 2012). Aphidophagous coccinellids tend to lay eggs nearby aphids and their voracious larvae actively seek out colonies of aphids, especially the very mobile final instar (Ferran and Dixon, 1993, Sattar et al., 2008, Hodek and Evans, 2012). As adults, predatory ladybirds have powerfully-built mandibles that crush and tear their prey, but their consumption rates can vary with sex and reproductive stage, relative size of both predator and prey, prey density, plant surfaces, temperature and humidity (Hodek and Evans, 2012, Honěk, 2012). Considering the efficiency of foraging, ladybird searching was once commonly believed to be a random process (Hagen, 1962). However, although the sensory abilities of ladybirds are still relatively poorly understood (Ferran and Dixon, 1993, Hodek and Michaud, 2008), chemical cues, visual cues, locomotor activity, and plant architecture are now thought to be important factors for foraging movements and prey location (reviewed by Pervez and Yadav, 2018). Ladybird searching behaviour is discussed in more detail in Chapters Seven, Eight and Nine.

The life cycle of ladybirds usually lasts around one year (AHDB, 2015) and, as with other holometabolous insects (those with complete metamorphosis), development starts with an egg, which hatches into a larva that goes through four instars, before pupation and metamorphosis into an adult (Dixon, 2000). Larval development of aphidophagous ladybirds occurs on aphid-infested stands of field crops, wild herbs, shrubs or trees, with young adults then feeding on both insect and vegetable food (Honěk et al., 2007). The ability of ladybirds to reduce aphid populations has led to ladybirds playing an important role in the development of biological control (Hagen, 1962). Due to their relatively fast development and high consumption rates, ladybirds are often more effective predators than other generalist predators, such as lacewings (Hindayana et al., 2001, Alhadidi et al., 2018).

The family Coccinellidae comprises roughly 6000 species (Seago et al., 2011), but the species primarily used in this thesis – seven-spot ladybird, *Coccinella septempunctata* and two-spot ladybird, *Adalia bipunctata* – are two of the most common found in Britain (Roy et al., 2012). As its name suggests, the seven-spot ladybird is typically characterised by a red elytra with seven black spots (see Figure 2.2). *C. septempunctata* is thermophilic, found mainly on herbaceous plants, and is the most common ladybird in Europe (Ferran and Dixon, 1993, Dixon, 2000, Hodek and Michaud, 2008). *C. septempunctata* is an important biological control agent, feeding on many different species of aphid (Rana and Kakker, 2000, Sattar et al., 2008), but also other insects, fungal spores, and pollen (Triltsch, 1997). There are, however, cases of attempted biological control where it has subsequently decimated native coccinellid populations (Elliott et al., 1996). Like *C. septempunctata*, the two-spot ladybird also lives up to its name sometimes, but it is a highly polymorphic species occurring in many forms ranging in colour from red to black (Omkar, 2005). *A. bipunctata* is typically a tree-dwelling species (Omkar, 2005), but can be found foraging on weed and vegetable plants (Sengonca et al., 2002) or other shrub and herbage habitats (de Jong et al., 1991, Ferrer et al., 2016). *A. bipunctata* feeds on many aphid species as well as coccids, diapsids, and pollen (Omkar, 2005), but has been found to be a relatively poor biological control agent. Despite some limited successes when part of combined releases with other natural enemies, efforts have often failed due to unfavourable conditions such as poor weather, male-killing symbionts, and victimisation by avian and coleopteran predators (Omkar, 2005).

Both *C. septempunctata* and *A. bipunctata* are polyphagous and generalist, eating a wide range of aphid species encountered in their habitats: *C. septempunctata* can feed on at least 24 aphid species (Hodek and Michaud, 2008), while *A. bipunctata* can feed on at least 50 aphid species (Ferrer et al., 2016). For different aphid species, the relative predatory efficiency of seven-spot and two-spot ladybirds can differ (Deligeorgidis et al., 2011), but both have been reported to predate pea aphids (Alhadidi et al., 2018, Alhadidi et al., 2019) and potato aphids (Walker et al., 1984, Sengonca et al., 2002).

# Chapter 3: Dropping as an antipredator defence

Material from this chapter formed the basis of the publication: Humphreys, R. K. & Ruxton, G. D. 2019. Dropping to escape: a review of an underappreciated antipredator defence. Biological Reviews, 94(2), pp.575-589. However, I have updated material in the light of new publications and developments in my vision of this field.

Dropping is a common antipredator defence that enables rapid escape from a perceived threat. However, despite its immediate effectiveness in predator–prey encounters (and against other dangers such as a parasitoid or an aggressive conspecific), it remains an underappreciated defence strategy in the scientific literature. Dropping has been recorded in a wide range of taxa, from primates to lizards, but has been studied most commonly in insects. Insects have been found to utilise dropping in response to both biotic and abiotic stimuli, sometimes dependent on mechanical or chemical cues. Whatever the trigger for dropping, the decision to drop by prey will present a range of inter-related costs and benefits to the individual and so there will be subtle complexities in the trade-offs surrounding this defensive behaviour. In predatory encounters, dropping by prey will also impose varying costs and benefits on the predator – or predators – involved in the system. There may be important trade-offs involved in the decision made by predators regarding whether to pursue prey or not, but the predator's perspective on dropping has been less explored at present. Beyond its function as an escape tactic, dropping has also been suggested to be an important precursor to flight in insects and further study could greatly improve understanding of its evolutionary importance. Dropping in insects could also prove of significant practical importance if an improved understanding can be applied to integrated pest-management strategies. Currently the non-consumptive effects of predators on their prey are underappreciated in biological control and it may be that the dropping behaviour of many pest species could be exploited *via* management practices to improve crop protection. Overall, this review aims to provide a synthesis of the current literature on dropping and to raise awareness of this widespread behaviour. It also seeks to offer some novel hypotheses and highlight key avenues for future research, some of which are explored further in Chapters Four, Five, Six, and Seven.

## 3.1 Introduction

One of the simplest ways for an organism on a raised substrate or in the air to escape an approaching threat is to drop. Dropping behaviour immediately removes an individual from the perceived hazard – be that a predator or another source of danger (such as a parasitoid or aggressive conspecific) – at least temporarily. Broadly, as an antipredator defence, dropping is a behaviour that could literally mean the difference between life and death for the prey. In the natural world, where organisms are engaged in an ongoing ‘struggle’ to survive and proliferate to pass on their genes to subsequent generations, any evolved adaptations that increase the likelihood of survival and/or breeding opportunities will be of advantage to an individual. Given that antipredator adaptations occur in almost every major taxonomic group and in every biome of the world, predation is clearly a selective force with great ecological and evolutionary significance (Ruxton et al., 2018, Sugiura, 2020). As discussed in Chapter One, antipredator adaptations can be morphological, chemical, or – as in the case of dropping – behavioural in nature, but all have evolved to reduce the risk of predation and, ultimately, mortality, thereby increasing the fitness of the prey. In many cases, when faced with a predatory threat, a prey species has multiple antipredator adaptations they could utilise. Some morphological defences, for example camouflage, may be deployed constantly, but where prey have the option to select a behavioural defence in response to an imminent threat they ought to select the behaviour that will best increase their fitness (balancing the costs and effectiveness of alternative behaviours).

I define dropping as a voluntary antipredator defence whereby a prey individual uses gravity, wind or water currents to power escape from imminent threat. Dropping can either be passive, where an individual simply falls away or releases its hold on a substrate (Dixon, 1958, Brown, 1974, Hajek and Dahlsten, 1987), or active, where the individual may jump away from a substrate (Haemig, 1997), sometimes kicking or somersaulting in the process (Brown, 1974). Whether passive or active, key to dropping is that the behaviour must result in the individual escaping in a trajectory determined primarily by the external force (gravity or bulk fluid flow) only modified modestly, if at all, by the organism itself.

This deceptively simple, but in fact very complex, behaviour is common and widespread as an antipredator defence, but (perhaps due to its lack of required morphological adaptations) it is currently understudied. As a key antipredator defence, dropping has significant consequences for both prey and predators at both individual and population scales. Undoubtedly, the precise cost–benefit framework surrounding dropping depends on the point of deployment within the predation sequence. Interactions between predators and their prey can be usefully broken down into a sequence of stages comprising: (1) encounter (spatial and temporal proximity), (2) detection, (3) identification, (4) approach, (5) subjugation, and (6) consumption (Endler, 1991, Caro, 2005). In the literature, antipredator defences employed by prey during stages 1–4 (ahead of subjugation) are referred to as 'primary defences', serving to influence the likelihood of the predator physically contacting the prey. So-called 'secondary defences' act once subjugation or contact has begun (stages 5 and 6). Unusually, dropping escape can be deployed either as a primary defence (see Brown, 1974, Clegg and Barlow, 1982, and Barnett et al., 2017 for some examples of dropping pre-subjugation) or a secondary defence (see Cloudsley-Thompson, 1995, and Castellanos et al., 2011 for some examples of dropping post-contact), suggesting that the timing of this escape behaviour can be varied in an adaptive way. Generally, we might not expect prey to drop as soon as they perceive a predator as they will not definitely be at risk of attack unless the predator has already detected them, identified them as prey and begun their approach. In many circumstances there will potentially be significant costs associated with dropping, for example, if there are other predators foraging below the prey's initial position, and so it may be adaptive to delay escape – and avoid the costs of dropping altogether – until a predation attempt is undoubtedly imminent. However, while dropping earlier in the predation sequence leads to more frequent dropping in prey, dropping later in the predation sequence runs the risk that a predator is successful in attacking before the prey gets a chance to drop, or that the predator can track and pursue prey that have dropped more readily. I expect the timing of dropping responses to specific predator attacks to be context-dependent, an idea which is explored in more detail in Chapters Five and Seven.

This review seeks to highlight the broad taxonomic distribution of dropping as an escape tactic, ranging from relatively passive undirected dropping using gravity, water, or wind, to relatively active and directed dropping in species that employ a form of gliding. I then detail a range of situations that can trigger the behaviour, considering visual, tactile, auditory, chemical, and parasitism-related triggers as well as the importance of multiple cues and abiotic factors in some situations. The costs, benefits, and trade-offs to dropping for both prey and predators are then discussed, including its use in combination with other defence mechanisms, such as tonic immobility (or 'death-feigning'), or cost-reducing behaviours –

such as silk drop-lines (Tolbert, 1975, Fitzpatrick et al., 1994, Castellanos and Barbosa, 2006, Sugiura and Yamazaki, 2006, Johnson et al., 2007) or aerial righting (Ribak et al., 2013, Meresman et al., 2017, Kane et al., 2021). This review also aims to draw attention to the importance of this underappreciated antipredator defence, not only for the individual organisms and populations affected, but also because of its potential to improve our understanding of the evolution of insect flight (Dudley et al., 2007, Yanoviak et al., 2009, Dudley and Yanoviak, 2011) and how improved knowledge of dropping could be applied practically to benefit ecological modelling and agriculture. Finally, I offer a number of outstanding questions that could stimulate fruitful future research in this area.

## 3.2 Taxonomic distribution and forms of dropping

As an antipredator defence that does not necessarily require specialist morphological adaptations or complex display behaviours, dropping is commonly used as an escape tactic across a wide range of taxa. However, from simple, undirected dropping dependent on gravity, to more controlled active dropping, and dropping in underwater contexts, there is a range of forms dropping can take to ultimately achieve the same antipredator function.

### 3.2.1 Release and undirected dropping

Often examples of terrestrial dropping escape consist straightforwardly of a release from a raised substrate and an undirected fall to whatever lies beneath. In many cases, an approaching threat may be aerial in nature, as is the case for several Mediterranean lizard species that respond to perceived avian predators by dropping from bushes to the ground (Vitt et al., 2002). Interestingly, though, some birds themselves – such as certain African bulbuls (*Pycnonotus barabatus* and *Andropadus latirostris*) and the frugivorous speckled mousebird (*Colias striatus*) – have also been reported as ‘dropping like rocks’ when they have been perched in trees (Lima, 1993, Caro, 2005). By so doing they escape into dense, underlying vegetation in order to avoid attacks from raptors.

Undirected dropping involving release from plants has been undoubtedly best studied in insects, however, and these small-bodied taxa can potentially encounter threats approaching from above, below or from immediate surroundings. The wealth of study of insect dropping is likely due to the behaviour’s prevalence in insects and the relative ease of study in these taxa. Most commonly, aphids are the focus of dropping research (Losey and Denno, 1998a, Francke et al., 2008, Wyckhuys et al., 2008, Gish et al., 2011, Gillespie and Acheampong, 2012, Ma and Ma, 2012, Ünlü et al., 2020b), but lepidopterans are also well known for dropping – most commonly in their larval stages (Perović et al., 2008, Castellanos and Barbosa, 2011, Greeney et al., 2012, Zhou et al., 2017), but also as adults (Honma et al., 2015, Wilkie, 2019). Important predatory insect taxa, including ladybirds (Lucas et al., 1997, Ben-Ari and Inbar, 2013) and spiders (Tolbert, 1975, Jackson et al., 1993, Cloudsley-Thompson, 1995, Blackledge and Pickett, 2000, Uetz et al., 2002), also drop from raised positions to escape predation themselves.

### 3.2.2 Controlled terrestrial descent

Like lizards and smaller birds, many species of primate – which despite being relatively large taxa are not apex predators – experience predation threats from raptors. Here dropping from tree canopies (sometimes with the help of vines) into lower branches or undergrowth can serve as an effective defence (Wright, 1998, de Souza Martins et al., 2005, Lledo-Ferrer et al., 2009, de Luna et al., 2010, Barnett et al., 2015, Barnett et al., 2017). To avoid injury, the descent will likely be more controlled in these larger taxa than in insect species – some of which may well possess hard integuments that help them withstand dropping – such that individuals plan their route down to some extent. Compared to smaller taxa, though, it may be that dropping in primates is a less costly behaviour in terms of the energy and time expenditure required for returning to suitable locations once a threat has passed. Often primate dropping behaviour is accompanied by alarm calls (see references above), but sometimes such warning calls occur in response to species that only resemble predators (Barnett et al., 2018). This has been suggested to occur in such species as the red-nosed cuxiús (*Chiropotes albinasus*) because although cautiously reacting to a ‘pseudopredator’ may reduce the time available for foraging or other activities, fitness is enhanced overall by not risking becoming a potential predator’s meal (Barnett et al., 2018).

### 3.2.3 Sudden loss of powered flight

Not all prey species that exploit gravity when dropping in an undirected way start off based on a substrate. For many insects fully capable of flight, dropping from the air suddenly during a bout of powered flight remains an important antipredator defence against flying predators such as bats (see Miller and Surlykke [2001] and references therein). For example, free-flying green lacewings (*Chrysopa carnea*) fold their wings and passively nose-dive when attacked by bats (Miller and Olesen, 1979); and bushcrickets (*Neoconocephalus ensioer*) have been found to show similar behaviours in response to ultrasound, suggesting that they might also drop from the air to avoid predation by bats during flight (Libersat and Hoy, 1991). Several bird species have also been reported as dropping out of the air to escape predation. Beyond simple escape dives, where small passerines often sharply pull up after dropping vertically over sea to evade falcons (Hedenstrom and Rosen, 2001), sometimes birds do completely drop with the help of gravity to reach refuge in water. Belted kingfishers (*Megaceryle alcyon*) have been observed suddenly plunging down into water when being pursued by hawks (e.g. Cooper's hawk, *Accipiter cooperii*, and sharp-shinned hawks, *Accipiter striatus*) over a river (Johnson, 1925, Skinner, 1928, Kirby and Fuller, 1978); in this case, dropping appears to be the first stage in the overall escape strategy as the kingfisher rapidly rises back out of the water facing a completely different direction and speeds off, meanwhile the hawk must attempt to arrest its momentum and readjust its attack direction. Where there is no body of water below, and only hard ground, some birds still escape mid-air attacks through dropping. Mourning doves (*Zenaida macroura*) and European starlings (*Sturnus vulgaris*) both perform sudden breast-first plunges into the ground to escape predation, but the substantial risk of injury associated with this tactic suggests it may be a truly 'last-ditch' defence (Lima, 1993, Caro, 2005).

### 3.2.4 Directed aerial descent and gliding

Many arboreal vertebrates have extended simple dropping escape behaviour into directed aerial descent (gliding at steep angles) or 'classical gliding' (gliding at shallow angles), including some species of lizard (Mori and Hikida, 1994, McGuire and Dudley, 2005, Dudley et al., 2007), frogs (Emerson and Koehl, 1990, McCay, 2001) and even snakes (Socha, 2002). These descents are slower than simple undirected release from a given substrate, making them a more controlled form of dropping. Many small mammals also use classical gliding (Jackson, 2000, Jackson, 2012), both for escape and travel functions. Classical gliding in terrestrial vertebrates was likely an important precursor to the evolution of powered flight (Dudley et al., 2007, Dudley and Yanoviak, 2011). Directed aerial descent has only fairly recently been described in a variety of wingless arboreal insects too (Yanoviak et al., 2005, Yanoviak et al., 2008, Yanoviak et al., 2011, Yanoviak et al., 2015, Zeng et al., 2015, Zeng et al., 2020). It is important to note here that, while dropping from the air and directed aerial descent fall under my definition of dropping, those species such as flying squirrels that have extensive morphological adaptations for gliding as part of routine movement through the environment as well as escape from predators are best seen as a separate phenomenon. Species well adapted to glide are able to modify the trajectory of falls significantly so that their dropping is less directly impacted by forces such as gravity.

### 3.2.5 Active drift

Having considered terrestrial and aerial dropping enabled primarily by gravity – but also potentially wind – it is also important to consider aquatic taxa that utilise water flow, sometimes alongside gravity, when evading predation. Many molluscan prey species in tidepools have been reported to escape from predaceous species, such as starfish and sea stars, by releasing their attachment to the substratum, flattening their mantles dorsoventrally, and gliding away in the surf (Bullock, 1953, Dayton et al., 1977, Hoffman, 1980, Lam, 2002). This escape behaviour strongly resembles directed aerial dropping but currently remains understudied. More appreciated in the literature at present is a similar behaviour that is seen in running waters, such as streams and rivers. Where water flows, benthic invertebrates can actively escape from their predators by releasing their foothold and entering the water column in a behavioural defence known as 'active drift' (Brittain and Eikeland, 1988). Active drift is one of several mechanisms of 'invertebrate drift' seen in running waters, a broader topic that has been the focus of many studies over the past few decades (see Brittain and Eikeland [1988], Wooster and Sih [1995], and Naman et al. [2016], and references therein). Active drift differs from most examples of terrestrial dropping, as the prey typically escapes *via* an upwards and horizontal trajectory rather than downwards. Nonetheless, I consider it a form of aquatic dropping due to its voluntary initiation, antipredator function, and its exploitation of an external force (here water currents rather than gravity) in the avoidance of an imminent threat.

Intriguingly, a different mechanism of dropping has been observed in conjunction with tonic immobility in two species of predatory cichlid fish – *Haplochromis livingstoni* and *Parachromis friedrichsthalii*. These fish appear to mimic a corpse by falling down through the water column and lying inert on the substrate as part of their hunting tactic (McKaye, 1981, Tobler, 2005). However, as an antagonistic tactic deployed to deceive potential prey, this behaviour certainly does not qualify for my definition of dropping as an antipredator defence.

The prevalence of dropping as an antipredator defence across such a wide range of taxa gives an indication of how effectively it must function as an adaptive and flexible escape behaviour, a key theme across this thesis. The evident convergent evolution of such a defence across multiple groups of very different animals demonstrates how important and fitness-enhancing behavioural adaptations can be, despite not necessarily occurring alongside specialist morphological adaptations. Due to the bulk of the dropping literature currently consisting of studies using insects this review – and, indeed, this thesis – will consequently focus on insect interactions with predators and parasitoids. However, work on other taxa is drawn in where possible and many of the broader concepts discussed and suggested research areas will apply equally to all species where dropping can influence survival and fitness. I hope to encourage further study of this conceptually simple but subtly complex defence within the context of all the predator–prey interactions in which it occurs.

## 3.3 Triggers of dropping

### 3.3.1 Visual and tactile cues

The most obvious trigger for antipredator dropping is the approach of a predator, following the prey's detection of the predator. In primates and other terrestrial vertebrates, approaching avian predators are often detected visually (Vitt et al., 2002, Lledo-Ferrer et al., 2009, de Luna et al., 2010, Barnett et al., 2017). Insect vision is less understood than primates', but several studies indicate that – despite their eyes' poor spatial resolution (Döring and Spaethe, 2009) – vision might play a role in aphids' predator detection process (Dixon, 1958, Hajek and Dahlsten, 1987, Gish, 2021). However, it is thought that substrate-borne vibrations are more commonly the most important modality for insects' detection of an approaching predator (Castellanos and Barbosa, 2006, Gish, 2021). Direct contact with a predator will also be an important trigger for dropping, and it has been found that the sensory hairs of *Orgyia leucostigma* (Lymantriidae) caterpillars enable stimulus-specific (and therefore predator-specific) responses depending on the velocity of hair-bending they experience (Castellanos et al., 2011). Specifically, *O. leucostigma* caterpillars predominantly drop in response to high hair-bending velocities – similar to those caused by more forceful, rapid predators like the wasp *Polistes fuscatus* and the spined assassin bug *Sinea diadema* – and predominantly walk away in response to low hair-bending velocities – similar to those caused by the slower-attacking stink bug *Podisus maculiventris* (Castellanos et al., 2011). I further investigate the influence of predator contact on prey propensity to drop in Chapters Five and Seven.

### 3.3.2 Auditory cues

Flying insects avoiding predation by bats use one main trigger to drop: detection of bat echolocation calls (Miller and Olesen, 1979, Miller and Surlykke, 2001). In a fascinating case of possible convergent evolution, Rosen et al. (2009) describe how females of the parasitic fly *Ormia ochracea* have evolved the same evasive behaviour as their cricket (*Gryllus rubens*) hosts, dropping towards the ground upon detecting bat echolocation calls. As disrupting flight by dropping could be energetically costly, some species of moth have evolved sophisticated abilities to discriminate between the calls of bats that are flying nearby looking for prey ('early attack') and calls of these bats that have detected prey and are moving into pursuit ('late attack') – this discrimination allows these moths to use only the truly threatening 'late attack' calls to trigger dropping behaviour (Ratcliffe et al., 2011, Corcoran et al., 2013). While some have suggested that prey species should benefit most by initiating defences as early as possible during predator–prey encounters (Endler, 1991, Fuiman and Magurran, 1994), this is a clear case where taking defensive action later on in the predation sequence can be more beneficial, corroborating the conclusion of Bateman et al. (2014) that there is no universal ecological or evolutionary advantage to defending early in the predation sequence.

### 3.3.3 Chemical and parasitism cues

Although chemical cues left behind by predators are known to influence the foraging decisions of various insect groups (Dicke and Grostal, 2001, Ninkovic et al., 2013), there is no evidence at present supporting the idea that insects detect the chemical cues of approaching predators in real-time, potentially due to the slow propagation of chemical cues in air (see Gish [2021] and references therein). However, in aphids a key trigger for dropping to avoid either predators or parasitoids is alarm pheromone, (E)-β-farnesene, released by conspecifics (Montgomery and Nault, 1977, Roitberg and Myers, 1978, Schwartzberg et al., 2008, Keiser et al., 2015, Harrison and Preisser, 2016). Parasitoids are important natural enemies of insects and it is thought that dropping may be the most common behavioural defence against them (Gross, 1993). Intriguingly, in aphids different symbionts may (Dion et al., 2011) or may not influence the likelihood of dropping in response to the presence of parasitoids (Lavy et al., 2015); in Chapter Four I discuss in greater depth, and contribute to, current understanding of the influence of symbionts on aphid defensive behaviours. Often in parasitoid–host encounters, dropping behaviour occurs as a response to – rather than in anticipation of – parasitism (Chau and Mackauer, 1997, Gillespie and Acheampong, 2012). Here, the trigger for dropping could relate to the physical external experience of parasitism or an internal chemical cue; future work could pick apart the trigger, or triggers, at play. It has been suggested that aphids altruistically commit ‘adaptive suicide’ when parasitized to protect uninfected kin (McAllister and Roitberg, 1987, McAllister et al., 1990), but this interpretation of observations and the methods used to generate them have been criticised (Latta, 1987, Tomlinson, 1987).

### 3.3.4 Multiple cues and abiotic factors

Information from multiple senses can be combined to trigger antipredator dropping, such as the detection of alarm pheromone release from conspecifics alongside vibrations caused by the movement of an approaching threat (Clegg and Barlow, 1982). Visual and vibrational cues have also been suggested to be integrated by aphids in a way that increases the accuracy of predator detection (Hartbauer, 2010, Gish, 2021). Fascinatingly, multiple cues have recently been recorded as key to triggering dropping in invertebrates escaping incidental ingestion by mammalian herbivores (Gish et al., 2010, Ben-Ari and Inbar, 2013). The combination of the heat and humidity of mammalian breath has been found to trigger dropping in coccinellid beetles (Ben-Ari and Inbar, 2013) and *Uroleucon sonchi* aphids (Gish et al., 2011). Additionally, pea aphid (*Acyrthosiphon pisum*) nymphs appear to combine breath cues with vibration cues to avoid erroneous dropping, which would be particularly costly at such a young life stage (Gish et al., 2012).

As well as biotic cues, abiotic factors may interact with triggers to dropping. Higher temperatures can increase predator foraging rate and, therefore, the vibrations sensed by prey (Brodsky and Barlow, 1986). Heat stress itself may (Ma and Ma, 2012) or may not trigger non-antipredator dropping in aphids, possibly depending on the clone or species studied (Stacey and Fellowes, 2002). In Chapter Seven, I explore the influence of environmental conditions on aphid dropping in response to predators, and in Chapter Six I test whether air temperature influences aphid behaviours on the substrate after antipredator dropping, but future work should explore the impact of heat stress further. There are clearly many potential triggers for escape dropping, and almost certainly there is yet more to discover about the combination of senses and cues utilised by non-insect taxa in particular in detecting approaching predators. Whatever the trigger for dropping, the costs, benefits and trade-offs associated with the behaviour will influence the decision to drop in prey and its impact on predators, whether they choose to pursue the prey or not.

## 3.4 Benefits, costs, and trade-offs associated with dropping

### 3.4.1 Prey perspective

#### *3.4.1.1 Benefits*

For prey, the most obvious benefit of dropping is the immediate escape from a threat. Dropping presents an immediately effective antipredator escape option and therefore in many situations it could offer the greatest benefit to prey fitness, relative to other tactics. As an example, Minoretti and Weisser (2000) found that pea aphids that try to walk away from seven-spot ladybird (*Coccinella septempunctata*) predators are often re-encountered by the predator. Dropping takes advantage of external forces – in this case, gravity – in order to exploit the path of least resistance, so that escape from enemies is simple and rapid. This benefit applies to avoidance of predators, parasitoids (Gross, 1993), and intra-guild predators where different instars of competing species vary in size and/or defences (Lucas et al., 1997, Sato et al., 2005, Raak-van den Berg et al., 2012). A subtler benefit of dropping in all circumstances where prey make their escape from a particular resource, for example a feeding area on a plant, may be that the prey individual subsequently locates a better resource, for example a feeding area on a plant of greater quality, but this is an example of a more complex, long-term benefit that is at present understudied.

#### *3.4.1.2 Costs*

Despite any immediate and long-term benefits, dropping comes with a suite of fitness costs. These can include energetic costs such as temporary distancing from resources, energetic loss while locating a subsequent resource, or selecting a poorer subsequent resource than the original. For insects such as aphids, reduced feeding time likely damages larval fitness (Johnson et al., 2007), increases development time for nymphs and is thought to reduce reproductive capacity in adult life (Agabiti et al., 2016). Even where aphids at any life stage are successful in locating a new plant on which to feed after dropping, their lifetime fecundity is likely to be impaired due to the loss of feeding time and energy expended in searching (Roitberg et al., 1979, Nelson, 2007). By reducing fecundity, this key ‘non-consumptive effect’ of predators – and parasitoids (Fill et al., 2012, Ingerslew and Finke, 2017) – can substantially reduce prey population growth (Nelson et al., 2004, Nelson and Rosenheim, 2006, Nelson, 2007, Culshaw-Maurer et al., 2020). In Chapter Six I examine and discuss the significance of several post-dropping behaviours with respect to the subsequent fitness of aphids, and in Chapter Ten I consider the broader, long-term implications of dropping for populations in greater detail.

For dropped individuals, the risk of mortality may also increase through: (*i*) exposure to new predators (Winder, 1990, Losey and Denno, 1998a, 1998b), (*ii*) exposure to harsh or harsher environmental conditions (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979, Perović et al., 2008), or (*iii*) time and energy spent finding a suitable resource post-dropping. There is also the additional risk of simply being pursued and consumed by the original predator. Strikingly, from a population perspective, incidents of dropping can lead to important changes in the spatial organisation of prey (Minoretti and Weisser, 2000, Fievet et al., 2007, Winder et al., 2014) and it is likely that such changes may increase the susceptibility of remaining prey to future attacks (Agabiti et al., 2016).

#### *3.4.1.3 Cost-reducing mechanisms*

There are clearly significant costs to dropping as an antipredator defence, particularly for undirected forms of dropping, and so it is no surprise that some species have evolved mechanisms to reduce these costs. Wingless pea aphids have recently been observed exhibiting an aerial-righting mechanism, whereby they assume a stereotypic posture when dropping that rotates them to a stable orientation. This improves their chances of clinging on to leaves that they encounter as they fall, thus lowering the likelihood of encountering risks to mortality on the ground (Ribak et al., 2013, Meresman et al., 2017). Recently, spotted lanternfly (*Lycorma delicatula*) nymphs have also been reported as exhibiting mid-air righting postures, however, this seems to constitute only one aspect of their strategy to minimise the cost of dropping as they also show reorientation while bouncing after contact with an underlying surface, and terrestrial righting after coming to a rest (Kane et al., 2021). Aphids' ability to re-cling to underlying plant material is a concept that is explored more fully in Chapters Six and Seven. Similarly, lizards such as *Anolis carolinensis* use their tails as a mid-air stabliser when jumping and falling to allow for coordinated landing on small branches after escaping predators. However, sometimes lizards will lose their tails by autotomy as a separate defence mechanism (Bateman and Fleming, 2009), and until their tail grows back stability when falling is compromised (Gillis et al., 2009). Lizards with autotomised tails, therefore, likely face greater potential risks when dropping and so must include their tail loss in decisions regarding their choice of antipredator behaviours.

A more commonly observed cost-reducing tactic than aerial righting in insect taxa is the production of silk thread ‘drop-lines’ – also known as ‘draglines’ (Tolbert, 1975, Blackledge and Pickett, 2000) and ‘life-lines’ (Sugiura and Yamazaki, 2006). Lepidopteran larval dispersal behaviour often involves the use of drop-lines in ‘silking’ and subsequent ‘ballooning’ on the wind (Terry et al., 1989, Cox and Potter, 1990, Zalucki et al., 2002, Moore and Hanks, 2004), but drop-lines are also important for avoiding threats (Castellanos and Barbosa, 2006). After dropping a short distance with silk to avoid a predator, larvae may continue to drop further (Johnson et al., 2007) or lose contact with the plant because of environmental factors such as heavy rainfall (Kobori and Amano, 2003, Chen et al., 2019) or strong winds (Perović et al., 2008, Chen et al., 2018). Alternatively, sometimes larvae climb back up drop-lines once the perceived threat has passed, thus avoiding the costs associated with losing their original position (Fitzpatrick et al., 1994, Sugiura and Yamazaki, 2006). The strength of physical disturbance represented by environmental factors and the likelihood of larvae reascending successfully can vary between species, in part due to their different levels of movement exhibited between feeding bouts and the differing strength of attachment to leaves offered by the silk structures they produce (Chen et al., 2018, Chen et al., 2019).

However, some parasitoids have evolved a remarkable countermeasure to drop-lines, whereby they locate the silk support line and slide down to their prey (Yeargan and Braman, 1986). More incredibly, Yeargan and Braman (1989) describe how the hyperparasitoid *Mesochorus discitergus* overcomes the dropping defence of green cloverworm (*Plathypena scabra*) in order to oviposit in the larval primary parasitoids inside already-parasitised caterpillars. Here, where a green cloverworm larva hangs from a leaf on silken threads, the hyperparasitoid usually hangs by its hind tarsi from the edge of the same leaf before reeling in the caterpillar, by pulling upward on the silken thread. The hyperparasitoid females then distinguish between green cloverworms parasitized by one of their hosts, *Cotesia marginiventris*, and those that are unparasitized, holding and probing parasitized ones further (Yeargan and Braman, 1989). Even where prey do not face parasitoid countermeasures, potential drawbacks to drop-lines may include silk production costs, the risk of strong winds (Perović et al., 2008, Chen et al., 2018), or the presence of flying predators. Not all silk-producing insects use drop-lines when dropping, so it is likely that related costs – such as dangling exposed to flying predators in mid-air – are less worth risking for some species in some situations. A detailed look at how silk-spinning ability and the potential for alternative defensive behaviours affect willingness to drop as lepidopteran larvae develop would be valuable.

#### *3.4.1.4 Trade-offs surrounding the decision to drop*

Whether prey have cost-reducing mechanisms to employ or not, the decision to drop will be guided by a number of important trade-offs concerning the relative benefits and costs of dropping to escape any perceived threat. Abiotic factors may influence the decision to drop even when they themselves are not the immediate trigger for dropping; for example, the daily cycle and illumination have been suggested to influence colonisation of plants in potato aphids (*Macrosiphum euphorbiae*) (Narayandas and Alyokhin, 2006) and so may influence mortality risk post-dropping. Similarly, high soil temperatures can prove fatal to dropping insects (Broadbent and Hollings, 1951, Ruth et al., 1975, Perović et al., 2008) and so the heat of the surrounding environment must be traded off with the immediacy of predatory danger. Abiotic factors such as temperature and light may be more likely to influence the fitness of insect taxa than larger taxa that utilise dropping, as smaller taxa experience greater variation between microclimates and, proportionally, drop a much greater distance (that will require more energy to recover a feeding location from) relative to their body size. Pea aphids have been described as ‘assessing’ risk and are less likely to drop when their environment is hot and dry (Dill et al., 1990). In Chapter Seven I explore the influence of some environmental conditions on the dropping propensity of wild aphids faced with predators, and in Chapter Six I test whether air temperature influences potato aphid behaviours on the substrate after dropping. Of course, any assessment of a potentially risky situation needs also to include a number of biotic factors.

Traits of an individual’s starting location or substrate itself should undoubtedly feed into any decision to drop, where prey leaves a desired resource during escape. If a resource is high quality it would be costly to abandon it for potentially lower-quality resources and individuals are less likely to drop from it (Dill et al., 1990). For insects, dropping likelihood may also depend on where the individual is located on a plant – Clegg and Barlow (1982) suggest that the stems of plants may be more dangerous for aphids than the undersides of leaves. The architecture of a plant may also influence the trade-off between danger and dropping if it influences how likely a dropped individual is to land on a lower part of the plant. In Chapters Six and Seven I explore in more detail both the influence of the plant position aphids are feeding on on the occurrence of antipredator dropping and some traits of plants that may make re-clinging to plant material post-dropping more likely. It would be interesting for future studies to investigate further, under more controlled conditions, whether herbivores select particular types of plants or particular places on plants on which to feed in part because such site selection improves the effectiveness of dropping as an antipredator strategy. Defensive morphological adaptations or other structural components of a plant may additionally help prey avoid predators or parasitoids (Obermaier et al., 2008), reducing the necessity of escape and therefore the frequency of dropping defence. An interesting additional consideration for insect prey species is whether plants are attended by ants or not; myrmecophilous aphids, for example, appear to be more dependent on ants for protection from predators than their own defensive tactics such as dropping (Nault et al., 1976, Suzuki and Ide, 2007).

Alongside plant factors, where relevant, traits of the prey themselves will influence their decision to drop. If dropping from a feeding resource, whether prey are specialist or generalist feeders could have important implications for their likely success at finding suitable locations at which to feed post-dropping (Castellanos and Barbosa, 2011). For insects requiring particular host plants, host-finding and dispersal abilities will be very important (Bierzychudek et al., 2009, Ben-Ari et al., 2015). Of course, host-finding abilities may be linked with plant factors, but there is still much to learn about how insects locate their host plants (Döring, 2014) and more studies regarding host-finding abilities may develop our understanding of the costs of dropping under different conditions. Life-history traits and prey state have also been found to influence the benefits and costs that will accompany dropping for prey individuals. In a theoretical model, *Uroleucon jacea* aphids with high relative gonadal investment or poor somatic energy states feeding on *Centaurea jacea* are predicted to be less willing to drop in response to predator attack due to their low survival probabilities when food uptake stops (Stadler et al., 1994).

In a similar vein, ontogeny could affect the trade-offs experienced by prey, but while adult or later-instar insect prey (Cornell et al., 1987, Losey and Denno, 1998a) are sometimes the more willing to drop, it is often the more juvenile stages in various insect taxa that drop most readily (Awan, 1985, Jackson et al., 1993, Cloudsley-Thompson, 1995, Lucas et al., 1997, Francke et al., 2008). Smaller lepidopteran larvae, for example, are perhaps less likely to be pursued by the original predator, but are also more susceptible to starvation through taking more time to reach a suitable feeding site post-dropping. If dropping is more costly to young, it would be adaptive for them to employ more sensory modalities to detect the level of threat. Gish et al. (2012), for example, found that young pea aphids avoid erroneous dropping when evading incidental ingestion by mammalian herbivores by dropping mostly in response to a combination of breath stimulus and vibrational stimulus. Ontogenetic differences in defence tactics are also seen in response to parasitism (Cornell et al., 1987, Chau and Mackauer, 1997). Willingness to drop at different life stages may be largely determined by relative vulnerability either to predators or climatic conditions (Perović et al., 2008). Additionally, while a couple of studies have suggested that prey density does not affect dropping behaviour in pea aphids (Losey and Denno, 1998a, Harrison and Preisser, 2016), Day et al. (2006) found that prey density was a significant influence on aphid dropping behaviour. Intraspecific differences, reproductive state, and associations with more intricate secondary defence mechanisms could usefully be explored further.

In part relating to some of the prey traits already mentioned, as well as more complex traits such as personality (Schuett et al., 2011), the trade-offs of dropping for prey are also thought to vary with species (Hajek and Dahlsten, 1987, Losey and Denno, 1998b), race (Kunert et al., 2010), strain (Zhang et al., 2016), and clone (Lowe and Taylor, 1964, Braendle and Weisser, 2001, Schuett et al., 2011, Schuett et al., 2015). Interestingly, kin recognition may influence dropping likelihood, even in non-social aphids (Muratori et al., 2014), but more work should explore this further.

For any species, dropping will not be the only defensive option and behavioural trade-offs will be made depending on any given situation. Dropping is often used in combination with other defence mechanisms and, for example, its deployment in conjunction with subsequent tonic immobility may be more effective against certain enemies than dropping alone, as has been suggested for invertebrate taxa such as spiders (Jackson et al., 1993, Blackledge and Pickett, 2000) and lepidopterans (Honma et al., 2015, Wilkie, 2019). The ‘decision’ to utilise dropping as an escape tactic may also depend on the potential for alternative behavioural adaptations or tactics in given situations (Ohno and Miyatake, 2007). For example, when under feeding stress pea aphids tend to kick at *Aphidius ervi* parasitoids rather than drop, to minimise the likelihood of energy shortfall (Villagra et al., 2002). In Chapter Five, I consider the relative use of antipredator dropping by aphids compared to a range of alternative behaviours, including walking and kicking. For birds that ‘choose’ to drop to the ground (Lima, 1993) or into water (Johnson, 1925, Skinner, 1928, Kirby and Fuller, 1978), it could be rationally assumed that the obvious escape method of flight is not always the wisest defence against larger and faster raptors. Environmental factors may also affect the behavioural trade-offs made, for instance prey may choose between dropping or startle displays depending on the underlying ground cover, with dropping only proving the more appealing option if there is somewhere to hide available below.

Distance to safe cover below may also influence the decision to drop, where the endpoint depends on gravity. From the perspective of avian species seeking to escape from pursuing predatory birds, Hedenstrom and Rosen (2001) analysed three aerial escape strategies and concluded that if prey are close enough to safe cover a vertical dive escape may be effective, even though smaller prey species will possess lower terminal diving speeds than that of their predators. Considering the influence of the underlying environment on insect prey, habitat complexity may also impact how easily dropped prey could locate new plants, how likely predators are to pursue dropped prey, and the effectiveness of dropping relative to other escape tactics; all of these factors deserve further study.

The type of predator may also determine the best defensive behaviour as, for instance, flight-capable insects may still choose to drop where their chances of flying escape are limited by dangers from above (Ben-Ari and Inbar, 2013). The predator:prey size ratio – often influenced by the instar stages of both sides – will also influence the effectiveness of running, kicking or dropping as alternative defensive strategies (Dixon, 1958, Brown, 1974, Evans, 1976a, Hoki et al., 2014). More generally, different predators have been observed to elicit different dropping rates in the same insect prey species (Losey and Denno, 1998a, Castellanos and Barbosa, 2006, Day et al., 2006, Castellanos et al., 2011). The influence of predator type on aphids’ defensive behaviours is a key aspect of the studies that I describe in Chapters Four and Five. Future research could manipulate the magnitude of perceived predatory risk and predator density to explore further the predatory triggers for dropping. But prey species are not alone in experiencing trade-offs in dropping situations; predators themselves will potentially experience benefits and costs that require decisions to be made regarding prey pursuit.

### 3.4.2 Predator perspective

#### 3.4.2.1 Benefits

When individuals drop they risk exposing themselves to new predators, and it is these new predators that can greatly benefit from dropping behaviour; instead of searching for and pursuing prey themselves, prey simply drops down (or indeed, flows downstream by active drift) to them. Interestingly, a laboratory study by Losey and Denno (1998c) showed that the combined predation rate of foliar-foraging (*C. septempunctata*) and ground-foraging (*Harpalus pennsylvanicus*) predators of pea aphids was almost double the sum of their individual predation rates when only one type of predator was present. The strength of the synergistic interaction between the predator types suggests that dropping behaviour elicited by foliar-foraging predators greatly benefits the ground-foraging predators and has great importance to the suppressive effect of predator complexes. In Chapter Ten, I highlight the role future studies might play in developing understanding of if, and how, post-dropping tonic immobility may mitigate the risk of predation by ground foraging predators.

#### 3.4.2.2 Costs and trade-offs surrounding the decision to pursue prey

For the predators that lose out on prey that have dropped to escape, the antipredator behaviour involves only costs. Predators will experience costs of reduced food uptake and foraging efficiency (Francke et al., 2008) and parasitoids can experience reduced fecundity (Niku, 1976). One way to reduce these costs may be to pursue dropped prey. Many insect-eating birds employ a ‘diving after’ behaviour to retrieve dropped prey items – although this has energetic costs, it can require less energy than seeking and acquiring new prey (Lohrl, 1978). Certainly, some insect predators do pursue their prey, for example, *Sceliphron caementarium* mud-dauber wasps vigorously pursue spiders that have dropped from their webs, and have been observed to crawl around under webs in gradually enlarging circular patterns to locate their fallen prey (Blackledge and Pickett, 2000). However, few studies have explored the trade-offs that may influence predators’ willingness to pursue dropped prey.

It might be reasonable to assume that prey size, and therefore energy content, would influence pursuit likelihood, with larger prey items being worthier of chasing. However, some predators may prefer to attack younger and smaller prey that are less able to defend themselves by kicking or running away, for example (Duran Prieto et al., 2016), so in some cases the opposite could be true. Some predators of aphids also prefer to attack particular colour morphs as well as size (Farhoudi et al., 2014), so this may also affect their pursuit likelihood. Of course, despite prey preferences, physical factors such as the distance dropped would likely have a significant bearing on the decision to pursue prey. Logically, distance would correlate negatively with pursuit likelihood, as the greater the distance dropped the more time and energy will be required for pursuit and, if the predator hunts in vegetation or in the air, for the subsequent return to typical foraging height. Another prey-related factor that could present a trade-off for predators considering pursuit may be the prey density remaining at the initial encounter point *versus* the density that has dropped away. I suspect that if there are numerous prey still available, a predator is unlikely to waste time searching for a dropped individual. This may be complicated, though, if a great number – perhaps the majority of a colony or group, for example – of prey items simultaneously drop (potentially triggered by conspecific alarm pheromone in the case of aphids). However, even in such a scenario, a predator may still be relatively likely to find alternative prey patches on the same plant without resorting to pursuing dropped prey. Future studies are needed to pick apart these complexities.

Alongside prey factors, I propose that predator state should influence the decision to pursue dropped prey. A predator's hunger state, general physical condition, and perhaps reproductive stage may affect its willingness to pursue a prey item and suffer any potential energetic costs in doing so. As well as the risk of not finding dropped prey, amounting to wasted time and energy, predators may also 'weigh up' the risk of encountering their own predators on an underlying substrate, or further downstream in the case of active drift. In some cases, predators may also just be too slow to feasibly 'chase' dropped food items. It may also be expected that some predators learn that certain types of prey in given situations are likely to drop and so these predators may alter their foraging strategies accordingly. As far as I am aware, there is a current dearth of knowledge about the trade-offs of dropping experienced from the predator's perspective and I would encourage researchers to pick up and explore some of the ideas suggested above.

## 3.5 Non-antipredator functions of dropping

The function of dropping behaviour may not be restricted to avoiding predators. When not being used to escape enemies, voluntarily falling is sometimes deployed by invertebrates as a shortcut to the ground or to access high-quality food patches (Haemig, 1997, Ohzora and Yano, 2011). In fact, more controlled dropping behaviour – known as ‘directed falling’ or directed aerial descent – has been reported in a number of wingless ant species (Yanoviak et al., 2005, Yanoviak and Dudley, 2006, Yanoviak et al., 2008, Yanoviak et al., 2010) as well as spiders (Yanoviak et al., 2015) and stick insects (Zeng et al., 2015, Zeng et al., 2020). Directed aerial descent is considered a form of gliding, but it occurs at steeper angles than ‘classical gliding’ (Dudley et al., 2007). Directed aerial descent appears to have evolved independently in multiple lineage of ants and, unlike gliding frogs for which the behaviour also has multiple independent origins (Emerson and Koehl, 1990), ant species in which directed aerial descent occurs do not show obvious external morphological differences from species within the same genus that do not exhibit directed aerial descent (Yanoviak et al., 2011). The occurrence of directed aerial descent in wingless insects suggests that insects have been engaged in controlled dropping behaviours prior to the origin of wings (Yanoviak et al., 2009, Dudley and Yanoviak, 2011). Selective pressures associated with remaining within an elevated foraging habitat may have motivated the antecedents to flapping flight from controlled dropping in lineages which are now volant (Dudley et al., 2007) and so, evolutionarily, dropping in primitive insects was likely an important precursor to insect flight (Hasenfuss, 2002).

Alongside the potentially vital role dropping may have played in the evolution of insect flight, it is important to consider the role dropping may have on the coevolution of natural enemies. Chau and Mackauer (1997) report how the parasitoid wasp *Monoctonus paulensis* preferentially attacks smaller, first-nymphal instars of pea aphids over larger and, in terms of resources for offspring development, more profitable instars in part because they were less at risk of dropping after successful parasitism. Dropping, as an antipredator defence, will presumably have impacted the foraging tactics of predators, as well as the host choice and oviposition behaviour of parasitoids, over evolutionary history and is likely to continue serving as an important behaviour in the ongoing arms race between natural enemies and their prey.

## 3.6 Practical applications for a better understanding of dropping

A greater understanding of dropping would be useful to integrate into models and studies of insect population dynamics (Agabiti et al., 2016), and – where dropping is costly to prey –

may also help to explain the invasion success of some predatory species (Raak-van den Berg et al., 2012, Hoki et al., 2014). Increased understanding of the complexities of dropping may be particularly important in the many cases where it is a common non-consumptive effect of natural enemies on pest species of agricultural crops (Cuny et al., 2019, Culshaw-Maurer et al., 2020). This could be of increasing importance across the globe as ongoing climate change is likely to influence the population dynamics of crop pests and their surrounding ecosystems (Michaud, 2010, Kambrekar et al., 2015, Wang et al., 2015). Of more practical use would be integrating insights about dropping behaviour into current pest management strategies (Culshaw-Maurer et al., 2020), aiming to mitigate some of the serious yield losses caused by some pests that drop (for example, aphids [van Emden and Harrington, 2007, Dedryver et al., 2010]).

Some insecticides are already thought to trigger dropping behaviour (Dixon and McKinlay, 1992), which could influence pests' subsequent growth, reproduction, and crop-damaging abilities. But, increasingly, purely chemical control of pests is problematic due to such issues as pests developing resistance (Dedryver et al., 2010, Springate and Colvin, 2012, Bass et al., 2014, Bass et al., 2015), damage to non-target species (Blacquière et al., 2012), other ecological issues (Geiger et al., 2010, Goulson and Kleijn, 2013, Sandhi and Reddy, 2020), and legislative restrictions. With the move away from chemical control, or at least to reduced chemical control, assisting the natural function of biological pest control could be an effective avenue for agriculture. Natural enemies are important and often effective controllers of crop pests (Chambers et al., 1986, Symondson et al., 2002, Schmidt et al., 2003) and the value of biological pest control to agriculture worldwide has been estimated at $417 billion per year (Costanza et al., 1997). However, the non-consumptive effects of 'biocontrol' natural enemies are currently underappreciated in the pest-control literature and drawing knowledge about dropping behaviour into pest-management strategies could provide some novel improvements.

Predators will suppress prey populations in part through the costs of induced defensive behaviours – such as dropping – alongside their direct consumptive effects (Nelson and Rosenheim, 2006, Culshaw-Maurer et al., 2020). Several studies have drawn attention to a synergistic effect – mediated by dropping – of foliar-foraging and ground-based predators controlling prey (Winder, 1990, Winder et al., 1994, Grez et al., 2011, Winder et al., 2014). Predator interactions could be particularly important in developing biological pest control

(Crowder and Jabbour, 2014), where it may also be the case that the role of parasitoids has previously been underappreciated (Schmidt et al., 2003). Of course, multiple predators may interact antagonistically (Meisner et al., 2011) and a range of other inter-related factors, such as the availability of alternative prey for generalist predators (von Berg et al., 2009), will complicate attempts to determine the overall effectiveness of pest control. But increased knowledge of dropping behaviour in predator–prey interactions could certainly help to draw out some of the more subtle impacts of predators and therefore help guide both the choice of biological control species – natural or introduced – and how to create the best conditions to prevent dropping escape behaviour giving pest species a fitness advantage, whether this advice relates to the predator involved, traits of plants, or environmental conditions. Intraguild predation is another important element to explore when aiming to develop integrated pest-management strategies (Rosenheim et al., 1995, Colfer and Rosenheim, 2001, Pérez-Valencia et al., 2019, Krey et al., 2021), and as Sato et al. (2005) point out, there is a need for studies exploring the fate of some predatory taxa once they have used dropping to escape intraguild predation.

The implementation of a biological control system factoring in dropping need not necessarily be used in isolation as a pest-management strategy (Sandhi and Reddy, 2020). While some types of chemical control seem to have long-term negative effects on natural biological control (Geiger et al., 2010, Krauss et al., 2011), this is not to say that certain chemical treatments cannot be used effectively in combination with biological control (Gentz et al., 2010). For any sustainable and safe pest-management strategy, a total systems approach is needed (Lewis et al., 1997), but incorporating findings about dropping into any management plan could only improve our understanding of how best to control pests while maintaining a thriving ecosystem.

## 3.7 Outstanding questions and opportunities for future research

Dropping is already known to be a widespread antipredator escape defence, but at present its importance is underappreciated and there is still much to learn. Considering both what may influence the decision to drop and what the consequences of dropping are for prey and their predators, there is a need for a greater exploration of abiotic factors, plant traits, variables in the underlying environment, prey factors and predator factors. In Chapters Four, Five and Seven of this thesis, I contribute to existing understanding on the influence of prey and predator factors on the occurrence of dropping by aphids, and in Chapter Six I present novel research into the influence of a range of environmental factors on what happens to aphids in the short term after dropping. Rather than repeating specific knowledge gaps already outlined in this chapter, or pre-empting more in-depth discussions from later chapters, I here draw attention to a few additional suggestions for future research that were beyond the scope of this thesis.

Ideally, well-designed field studies in natural conditions could be employed to investigate the impact of abiotic conditions on dropping behaviour, for insects in particular, such as weather and temperature. Such consideration of abiotic conditions may be particularly important for accurate assessments of prey behaviour moving forward, given that more extreme temperatures and weather events are predicted under climate change (IPCC, 2014, Chen et al., 2019, Devegili et al., 2019). Although the fieldwork described in Chapter Seven presents some initial findings on dropping by aphids in natural conditions, more extensive studies that take place under more variable conditions are required to determine the true impact of environmental variables. The influence of changing light conditions and diel period might be particularly well explored by field studies. Visual cues might be utilised by some taxa when detecting threats or locating suitable food sources post-dropping; Gish and Inbar (2006) suggested that future studies should address insect prey's ability to discriminate between visual cues, and time of day may well influence behavioural decisions. Diel period might also affect the foraging of predators, but some ladybird species continue foraging at high rates during darkness using volatiles of aphid-infested plants (Norkute et al., 2020). This suggests that aphids relying at least in part on visual cues to trigger dropping or locate hosts post-dropping may incur greater fitness costs during darkness, but predator-prey interactions overnight have been overlooked so far and it is unknown whether aphid sensitivity to other dropping cues or host-locating modalities shifts during darkness to compensate for reduced vision. The sensitivity of prey to environmental sounds, such as moving branches or rainfall, would also be interesting to explore through field studies, as reacting defensively to non-threatening noises would presumably incur needless fitness costs.

More generally, as laboratory results do not always reflect field studies on dropping or predator foraging (Braendle and Weisser, 2001, Latham and Mills, 2009, Raak-van den Berg et al., 2012), more field studies – or at least more natural design elements in experimental studies (such as real instead of artificial predators) – will shed valuable light on dropping behaviour. More studies with wild prey may also be especially important in assessing the behaviours that occur in the natural world, as laboratory culturing of some insects has been found to dramatically alter their typical responses (Thieme and Dixon, 2015). As touched on in section 3.6, dropping as a key non-consumptive effect of predators also needs to be included in more holistic studies of the potential small- and large-scale interactions (synergistic and antagonistic) between pest behaviours and practices involved in integrated pest management (IPM) (Culshaw-Maurer et al., 2020). Many major crop pests will utilise dropping, but the full effect of this in relation to biocontrol, chemical control and cultural practices needs to be carefully examined in working, agricultural fields in order to hone effective and sustainable management strategies (Culshaw-Maurer et al., 2020, Sandhi and Reddy, 2020, Krey et al., 2021). Increased understanding of specific cues for dropping by pests might also lead to the discovery of new management practices, for example, mimicking the vibrational cues of predators could trigger more dropping and/or habituate pests to the cues of actual approaching predators, thereby increasing predator consumption (see Gish [2021] and references therein).

Considering predators in particular, interactions between multiple predators should be explored further to see where predators act synergistically (Losey and Denno, 1998c, Krey et al., 2021) or antagonistically (Traugott et al., 2012) in their control of prey. It may be interesting to research whether there are any situations where foliar-foraging predators benefit from the presence of ground-foraging predators, although Losey and Denno (1998c) found no evidence of this. It is possible that, through using cues of different ground-based predators, studies may find that prey are sometimes less willing to drop despite also detecting foliar-based predators. Alternatively, prey may still drop but not all the way to the ground, or when they hit the ground they might flee to new plants with foliar-foraging predators on them in their hurry to escape from ground-foraging predators; these possibilities certainly warrant further investigation. More exploration of the frequency of predators pursuing dropped prey, and the factors that influence this decision, if it is a phenomenon that occurs, could also be valuable.

From a broader evolutionary perspective, any improved understanding of the behavioural ecology of dropping could help to explain why some species related to taxa that frequently utilise antipredator dropping either do not utilise this escape tactic themselves or use it far less commonly, e.g. bird cherry-oat aphids (*Rhopalosiphum padi*) (Long and Finke, 2014).

Learning more about the contexts that do and do not instigate dropping will help us pick apart the evolution of dropping as an effective antipredator tactic. This may also be relevant to ecological communities for which invasive species represent a threat to biodiversity. For example, some populations of pea aphid have been found to lack avoidance behaviours towards non-native ladybird predators, apparently due to an inability to recognise their chemical cues (Bertleff et al., 2020, Ünlü et al., 2020a). However, this does not necessarily lead to reduced dropping by aphids in response to invasive ladybirds, as vibratory cues associated with their presence can still elicit the escape response (Ünlü et al., 2020b). Naivete towards invasive species of predator, or parasitoid, may bring about reduced dropping in other circumstances with any prey taxa that drop, though, if the non-native natural enemy presents cues that prey have not co-evolved alongside; this could have striking knock-on effects for population and community dynamics.

Dropping behaviour may also be a key factor in the evolution of insect flight, and any work that builds on our understanding of that is at least as valuable as studies presenting mechanisms for the evolution of flight in pterosaurs or early birds; arguably, flight in insects is an even more essential topic to explore due to the vast quantities of flying insects that inhabit the planet. Overall, there is still a lot to learn about dropping, but to me the most exciting and untapped questions for this topic are: (1) What factors are key in influencing the occurrence and consequences of dropping behaviour, both in the short and long term? It is this question that I focus on in this thesis. (2) Why do some taxa that are physically capable of dropping as a defence tactic not utilise the behaviour against predators, i.e. what conditions over evolutionary time bring about dropping as a defence? (3) Do predators sometimes pursue dropped prey, and what factors influence this decision? (4) What role did dropping behaviour play in the evolution of insect flight? (5) How could dropping behaviour be effectively exploited as part of integrated pest-management strategies?

# Chapter 4: Influence of *Hamiltonella defensa* infection, predator type, and genotype-associated parasitism susceptibility on antipredator behaviours in pea (*Acyrthosiphon pisum*) and potato aphids (*Macrosiphum euphorbiae*)

Facultative endosymbionts can provide fitness benefits to their aphid hosts, but some partnerships can be ecologically costly. In the pea aphid (*Acyrthosiphon pisum*), infection with the *γ*-proteobacterium *Hamiltonella defensa* has been reported to confer resistance against parasitoids, but may also reduce the frequency of aggressive and escape behaviours exhibited in response to predators. In potato aphids (*Macrosiphum euphorbiae*), *H. defensa* does not influence susceptibility to parasitism, but its impact on antipredator behaviours remains unexplored. My study investigated defensive behaviours in two pea aphid lines (differing in *H. defensa*-infection status) and four potato aphid lines (that additionally differed in genotype-associated parasitism susceptibility) when faced with foraging ladybird (*Adalia bipunctata*) adults and lacewing (*Chrysoperla carnea*) larvae on intact plants. The experimental approach detailed in this chapter also forms the basis of Chapters Five and Six, but the focus on symbiont- and genotype-related effects is unique to this chapter. In response to ladybirds, symbiont-infected pea aphids exhibited proportionately fewer evasive defences (dropping and walking away) than cured pea aphids, though infected pea aphids exhibited more frequent aggressive kicking. Predator type, though, appeared to be the key predictor of pea aphid defences, with ladybirds provoking significantly more evasive, aggressive, and total counts of behaviours than lacewings. For potato aphids, symbiont status, predator type and aphid genotype (i.e. parasitism susceptibility) all influenced behavioural repertoires. However, infected aphid lines of both parasitism-resistant and parasitism-susceptible genotypes showed greater differentiation in behaviours in response to the two predator types than the uninfected lines. The presence of the symbiont *H. defensa* may be a key determinant of aphid defensive behaviours, rather than parasitism resistance more generally. Neither symbiont infection status nor genotype appeared to affect the number of individuals consumed by predators.

## 4.1 Introduction

As introduced in Chapter One, predation is a major selective force in nature, influencing many aspects of animal morphology, physiology, and behaviour. The threat of predation, and the associated fitness costs, has thus led to the evolution of diverse antipredator adaptations in prey species of all taxa (Edmunds, 1974, Harvey and Greenwood, 1978, Sih, 1987, Ruxton et al., 2018). In any given predator-prey encounter, a prey individual will usually have a range of behavioural defensive adaptations that they could deploy, but the behaviour ultimately exhibited can be influenced by many variables.

Aphids (family Aphididae) are phloem-feeding herbivorous insects that are a dietary staple for an array of generalist invertebrate predators; in Chapter Two I outlined the basic biology of aphids, see the Appendix for more details. While aphids were for a long time considered to be entirely helpless, thin-skinned, sedentary prey exhibiting no self-defence against predation (Imms, 1947), they are now appreciated as having a wide range of defensive behaviours (Dixon, 1958). Depending on species and circumstances, aphid antipredator behaviours to evade or deter predators include: remaining motionless to avoid detection (Dixon, 1958, Dixon, 1985, Brodsky and Barlow, 1986), kicking (Dixon, 1958, Hartbauer, 2010, Dion et al., 2011, Polin et al., 2014), attacking with frontal horns (Arakaki, 1989), backing-up and walking away (Dixon, 1958, Clegg and Barlow, 1982, Brodsky and Barlow, 1986), and dropping from plants to escape (Dixon, 1958, Roitberg and Myers, 1978, Clegg and Barlow, 1982, Agabiti et al., 2016, Harrison and Preisser, 2016). Such a portfolio of possible behaviours makes aphids ideal as candidates for the study of the factors that might influence behavioural responses to predators.

Antipredator behaviours in aphids are commonly triggered by direct contact with a predator (Roitberg et al., 1979, Dill et al., 1990, Nelson and Rosenheim, 2006), but can also be elicited by: visual stimuli (Hartbauer, 2010, Ben-Ari et al., 2014, Gish, 2021); the sensing of plant-borne vibrations from a perceived approaching threat (Clegg and Barlow, 1982, Brodsky and Barlow, 1986, Agabiti et al., 2016, Gish, 2021); the detection of alarm pheromone, (E)-$\beta$-farnesene, released by disturbed conspecifics (Montgomery and Nault, 1977, Roitberg and

Myers, 1978, Schwartzberg et al., 2008, Keiser et al., 2015, Harrison and Preisser, 2016); the detection of other environmental variables through different senses (such as the sensing of mammalian grazer breath by aphids avoiding incidental ingestion) (Gish et al., 2010, Gish et al., 2011, Ben-Ari and Inbar, 2013); or a combination of any of the above triggers (Clegg and Barlow, 1982, Gish et al., 2012, Ben-Ari and Inbar, 2014, Gish, 2021). Whatever the trigger, from the possible suite of defences aphids can exhibit, dropping from host plants to escape predators is thought to be widespread, common and effective response of aphids to perceived threats (Montgomery and Nault, 1977, Dixon, 1985, Gross, 1993). However, as discussed in Chapter Three, dropping to the ground can itself be a costly behaviour, given the resultant exposure of the aphid prey to potential risks of mortality: such as ground-foraging predators, high ground temperatures, or subsequent failure to locate a new, suitable host plant (Roitberg and Myers, 1979, Roitberg et al., 1979, Losey and Denno, 1998b, Gish and Inbar, 2006). The use of dropping relative to other potential defensive responses, therefore, should be expected to be highly context-dependent.

A range of abiotic and biotic factors have been shown to impact the antipredator behaviours elicited by aphids. Many studies have explored external factors that can influence the occurrence of dropping behaviour, including weather variables such as air temperature (Roitberg and Myers, 1978, Dill et al., 1990, Ma and Ma, 2012, Straw et al., 2019), variables relating to the host plant (Clegg and Barlow, 1982, Dill et al., 1990, Stadler et al., 1994, Matsubara and Sugiura, 2018), and variables relating to the predatory threat (Dixon, 1958, Brown, 1974, Evans, 1976a, Brodsky and Barlow, 1986, Losey and Denno, 1998a, 1998b, Day et al., 2006, Francke et al., 2008, Hoki et al., 2014, Tamai and Choh, 2019). Less attention has thus far been paid to properties of the prey that could influence use of dropping and other defensive behaviours in aphids, but species (Montgomery and Nault, 1977, Losey and Denno, 1998b, Nelson and Rosenheim, 2006, Grez et al., 2011, Ingerslew and Finke, 2017), clone (Lowe and Taylor, 1964, Braendle and Weisser, 2001, Stacey and Fellowes, 2002, Schuett et al., 2015, Fan et al., 2018), host-adapted race (Kunert et al., 2010, Ben-Ari et al., 2019), individual personality (Schuett et al., 2011), age (Roitberg et al., 1979, Losey and Denno, 1998a, Gish and Inbar, 2006, Matsubara and Sugiura, 2018), and physiological state (Villagra et al., 2002) have all been suggested to have impacts. One relatively new field of study involves consideration of how the presence of facultative endosymbionts might influence antipredator behaviour (Dion et al., 2011, Polin et al., 2014).

Endosymbionts are microbes that form associations with insects, such as aphids, and are located intracellularly in the host insect (Clark et al., 2010). Most aphid species possess the obligate (or 'primary') bacterial endosymbiont *Buchnera aphidicola*, which synthesises essential amino acids for its host (Douglas, 1998, Gündüz and Douglas, 2009), but they can also harbour additional facultative (or 'secondary') bacterial endosymbionts (Guo et al., 2017). Facultative endosymbionts are not essential for insect survival and are present at frequencies that vary between aphid populations (Smith et al., 2015). The phenotypic effects that different facultative bacterial endosymbionts have been documented to exert on their hosts are diverse and variable (Guo et al., 2017, Vorburger, 2018). Such effects (see Guo et al. [2017] for a review) can include influences on: body colour (Polin et al., 2015); resistance to fungal pathogens (though this may co-occur with high intrinsic resistance) (Hrcek et al., 2018); the effectiveness and/or abundance of other symbionts (Leclair et al., 2016, Leclair et al., 2017, Li et al., 2018); nymph growth (Leybourne et al., 2018); the production of winged offspring/wing polyphenism (Reyes et al., 2019); host survival and fecundity (Leclair et al., 2017, Niepoth et al., 2018, Reyes et al., 2019); susceptibility to insecticides (Skaljac et al., 2018); suppression of plant defences (Wang et al., 2020); interactions with other insect herbivore species (Hertag and Vorburger, 2018); attractiveness of plants to foraging parasitoids when fed on by infected aphids (Frago et al., 2017); and even host plant growth and resource allocation (Hackett et al., 2013, Bennett et al., 2016). Some effects are clearly beneficial to the aphid host (such as increased stress tolerance or protection against natural enemies) but some are detrimental (such as reduced fecundity and lifespan), and the same symbionts can have multiple ecologically-important effects (Heyworth and Ferrari, 2015); see Vorburger (2018) and references therein for a review of the phenotypic effects facultative bacterial endosymbionts have on aphids. There is much yet to uncover regarding the full role endosymbionts play in the fitness and ecology of their aphid hosts. What is evident is that different symbionts have differing phenotypic effects, but also that the same symbiont has the potential to impact its host positively and negatively to varying degrees depending on ecological context.

One symbiont that appears to affect both aphid resistance to parasitism and their behavioural responses is the facultative endosymbiont *Hamiltonella defensa*. *H. defensa* is a *γ*-proteobacterium that has been reported to provide resistance against *Aphidius ervi* parasitoid wasps in pea aphids (Oliver et al., 2003), apparently independent of host genotype (Oliver et al., 2005). Dual infection of pea aphids with *H. defensa* and the pea aphid X-type symbiont (known as PAXS) (Guay et al., 2009) or *Serratia symbiotica* (Oliver et al., 2006) is thought to confer even greater parasitism resistance (Donald et al., 2016). *H. defensa* also appears to be a source of protection against parasitism in some other aphid species, including bird cherry-oat aphids (*Rhopalosiphum padi*) (Leybourne et al., 2018), cowpea aphids (*Aphis craccivora*) (Asplen et al., 2014), and black bean aphids (*Aphis fabae*) (Schmid et al., 2012); though not against all species of parasitoid wasps (Cayetano and Vorburger, 2015). In fact, a recent study by Hafer-Hahmann and Vorburger (2020) suggests that parasite diversity is the key driver of diversity in *H. defensa* strains in the black bean aphid; the authors suggest that the phenomenon of natural enemies driving symbiont composition likely occurs generally in nature. However, *H. defensa* is also the symbiont that has been most heavily implicated thus far as an influence on aphid behaviour (Vorburger, 2018). Returning to studied pea aphid-*H. defensa* associations, it has been reported that pea aphids infected with *H. defensa* exhibit a reduced frequency of defensive behaviours in the presence of a parasitoid than uninfected ones (Dion et al., 2011). Such a reduction of defensive behaviours could theoretically be adaptive to the aphid if it enables them to avoid taking redundant and costly protective measures against parasitoid attack (Dion et al., 2011). However, even if infection with the symbiont provides protection against parasitism, any subsequent reduction of defensive behaviours could also carry a substantial cost if hosts are subsequently more vulnerable to other natural enemies (Sochard et al., 2020). Following this finding, Polin et al. (2014) investigated whether infection with *H. defensa* reduced expression of aphid defensive behaviours against predators as well, thereby increasing susceptibility to predation. They exposed four pea aphid clones free of any of facultative symbionts, three clones harbouring *H. defensa* alone and four clones doubly infected with both *H. defensa* and PAXS to predation by a ladybird on an excised leaf in a glass cage. Aphid aggressiveness (defined as quick motions of legs and/or body repelling the predator) and evasiveness (walking or dropping away) were recorded. Aphids hosting *H. defensa* alone or in association with PAXS were shown to suffer higher predation than uninfected ones, as infected aphids exhibited aggressive and evasive behaviour less frequently in presence of predators (Polin et al., 2014). However, the consequences of *H. defensa* infection on the behavioural responses of pea aphids feeding on live plants, rather than excised leaves, and aphids encountering predators with different foraging styles have not yet been tested.

It is also unknown whether the observed impact of *H. defensa* infection on the behavioural responses of aphids is due to the presence of the symbiont itself or its associated parasitism resistance. One means by which this could be explored is through the testing of aphid species that vary in their genotypic susceptibility to parasitism and/or for which *H. defensa* does not provide protection from parasitism. The findings of a study by Oliver et al. (2005) suggested that for pea aphids the strain of *H. defensa* determines the level of resistance it provides, not aphid genotype or any interaction between strain and genotype; at least for the strains and genotypes considered in their study. However, other species of aphid for which genotype may determine parasitism resistance more than *H. defensa* infection could be a useful means by which to explore the cause of behavioural changes. A study by Clarke et al. (2017) suggested that there was little evidence that *H. defensa* provided strong protection from parasitism in the potato aphid. Only lines from a single potato aphid genotype exhibited genotype-associated parasitism resistance. The lines in which *H. defensa* occurred also did not appear to experience the fitness costs sometimes associated with *H. defensa* infection (Oliver et al., 2006, Simon et al., 2011, Vorburger and Gouskov, 2011) or parasitism resistance; in fact they showed faster development, higher survival and greater fecundity (Clarke et al., 2017). Whether *H. defensa* generally does not significantly improve parasitism resistance in potato aphids, or whether this only applies to the particular strains typically found infecting potato aphids or the parasitoid species tested (*Aphidius ervi*), has not yet been investigated. Further, the relative use of different behavioural responses by potato aphids of parasitism-resistant or parasitism-susceptible genotypes with different *H. defensa* infection statuses during encounters with natural enemies have not, to my knowledge, been explored.

This current study aimed to address gaps in the literature concerning the impact that symbionts can have on the defensive behaviours exhibited by their hosts, which is an important topic given the significance of behaviour on the outcome of predator-prey encounters and prey fitness. Infection with facultative endosymbionts providing resistance to parasitism (*H. defensa* +/- PAXS) can make aphids defend themselves less frequently (by aggression or evasion) against parasitoid attack (Dion et al., 2011) or against predators (Polin et al., 2014), but this effect has only been tested under specific conditions thus far. My study builds on the work by Polin et al. (2014), by making three key methodological changes: i) rather than conduct the study on excised leaves, encounters with predators occurred on entire plants, creating a more realistic, whole-system setting in which behaviours could take place; ii) responses to two predator types, which differ in their foraging style, were explored; and iii) potato aphid lines were tested as well as pea aphid lines. In doing so, I sought to address three research questions:

1. Can the effects of *H. defensa* infection on antipredator defence be observed in pea aphids faced with predators on live plants?
2. Does symbiont infection differentially affect pea aphid behaviour against different predator types?
3. Is the effect due to symbiont presence per se or due to parasitism resistance generally?

This study exposed aphids to *C. carnea* lacewing larvae and *A. bipunctata* ladybird adult predators on live plants. Data collected during these experiments also form the basis of Chapters Five and Six, but the focus on symbiont- and genotype-related effects is unique to the aims, analysis, and conclusions of this current chapter. To address research questions 1 and 2, two pea aphid lines were studied, both of the same genotype but one naturally infected with *H. defensa* and the other cured of *H. defensa*. Any apparent differences in the lines' behavioural responses to predators could therefore be associated with *H. defensa* infection. To address research question 3, four potato aphid lines were studied, differing in both *H. defensa* infection status and in genotype (parasitism-resistant or -susceptible). *H. defensa* does not confer protection against parasitism in potato aphids (Clarke et al., 2017) so, for example, if the responses of potato aphids of both parasitism-resistant and -susceptible genotypes were affected by this symbiont in a similar way to pea aphids this might indicate that the behavioural consequence is due to the symbiont's presence itself rather than general resistance to parasitism. The implications of my findings for aphid biological control are considered briefly in the Discussion.

## 4.2 Materials and methods

Note that the details given in the sections on plant material, insect rearing and maintenance, and experimental protocol below are also relevant to Chapters Five and Six, though additional observations made and methodological steps taken specifically for the studies in these subsequent chapters are described in the appropriate 'Materials and methods' sections (5.2 and 6.2, respectively) later in this thesis.

### 4.2.1 Plant material

Plants were grown from seed (*Vicia faba* cv The Sutton) or tubers (*Solanum tuberosum* cv Désirée) in commercially produced insecticide-free compost mix (sand–perlite–peat mix containing N:P:K 17:10:15; William Sinclair Horticulture LTD, Lincoln, UK) in a glasshouse with supplementary light (16:8 h light and 20:15°C day:night) and watered daily. Immature bean plants 2-4 weeks from planting and immature potato plants 2-3 weeks from planting were used in experiments.

### 4.2.2 Insect rearing and maintenance

The aphid lines used in experiments were from established laboratory cultures: the pea aphid lines originated from wild lines collected in 2010 (supplied by Dr. Julia Ferrari, University of York) and the potato aphid lines originated from wild lines collected between 2014-2016 (supplied by the James Hutton Institute, Dundee). Aphid clonal lines were reared on excised leaf material in ventilated containers comprising one Perspex cup (50mm width x 150mm depth) placed inside another; plant material was inserted through a c. 5 mm circular hole in the base of the inner cup into c. 10 mm depth of water in the base of the outermost cup, and the cup surface was sealed with a mesh-ventilated lid. Pea aphids were cultured on leaves from 2-3-week-old faba bean plants and potato aphids were cultured on leaves from 3-week-old potato plants, with plant material refreshed weekly. Using this set up, age-synchronised cohorts of 3rd – 4th instar nymphs were produced daily for experiments; these were 6-7 d old for *A. pisum* and 8-9 d old for *M. euphorbiae*.

Adult two-spot ladybirds and lacewing larvae were supplied by Dragonfli (Essex, UK) and Ladybird Plantcare (East Sussex, UK). Predators were kept in large, ventilated Perspex cages (90cm H x 35cm W x 45cm D) housed within a Snijders Scientific MicroClima growth chamber maintained on a light:dark cycle of 16:8 h and 15:10°C, with a relative humidity of 60-70%. Dry paper towels provided non-slippery substrate and shelter, and predators were supplied daily with 2-3-week-old faba bean plants infested with an excess of pea aphids of a clonal line shown to be free of facultative bacterial endosymbionts (including *H. defensa*) and of a different genotype to experimental aphid lines. Ladybirds were additionally supplied with dilute sugar water-soaked cotton wool. Two days prior to assays, predators were transferred (along with pea aphid-infested leaf material) to ventilated containers and placed in the glasshouse where assays took place; predators were then isolated into paper-towel lined ventilated cups and starved for 24-32 h.

### 4.2.3 Insect material

Pea aphid lines were supplied by Dr. Julia Ferrari (University of York). They belonged to the same genotype, with one line possessing *H. defensa* (GT218-*H. defensa*) and one line having been artificially cured of *H. defensa* (GT218-cured), enabling comparison between aphid lines with identical genetic backgrounds, differing only in presence/absence of *H. defensa*. Confirmatory genotyping and facultative endosymbiont detection were conducted (see Table 4.1 and below).

**Table 4. 1:** Summary of the pea and potato aphid lines used in experiments.

| **Species** | **Line** | **Genotype** | ***H. defensa* present** | **Other symbiont present** | **Source** |
|---|---|---|---|---|---|
| ***A. pisum*** | GT218-cured | 218 | No | No | Julia Ferrari – Eling, Berkshire, *Medicago sativa*, collected 28/05/10 |
| | GT218-*H. defensa* | 218 | Yes | No | |
| ***M. euphorbiae*** | MW16/67 | 1 – parasitism resistant | No | unknown | Weir farm – site 4, Fife, 2016 |
| | RB15/11 | 1 – parasitism resistant | Yes | unknown | Invergowrie, 2015 |
| | MW16/48 | 2 – susceptible to parasitism | No | unknown | Perth, 2016 |
| | AK14/01 | 2 - susceptible to parasitism | Yes | unknown | Perth, 2014 |

Characterised potato aphids of known genotype and endosymbiont status were obtained from cultures held at the James Hutton Institute, Dundee. Two of the potato aphid lines had previously been found to be parasitism-resistant (termed ‘genotype 1’); of these, line RB15/11 was naturally infected with *H. defensa* when obtained from the wild while MW16/67 was not. The other two potato aphid lines belonged to a parasitism-susceptible genotype (genotype 2); of these, line AK14/01 was naturally infected with *H. defensa* when obtained from the wild while MW16/48 was not (see Table 4.1). After the experiment was completed, routine checks of the stock lines were carried out by Gaynor Malloch and Desiré Macheda at the James Hutton Institute (Dundee). DNA was extracted from the four potato aphid lines using a protocol adapted from Stanton et al. (1998). Seven microsatellite loci were used for genotyping (Raboudi et al., 2005, Clarke et al., 2017), with PCR and multiplex PCR reactions run following a thermocycler program based on that by Sloane et al. (2001). A ‘nested’ diagnostic PCR screening was then conducted to check for the presence or absence of *H. defensa* in the four potato aphid lines. After a first round of PCR with primers which amplify both the primary symbiont *Buchnera* and any secondary endosymbionts (Fukatsu et al., 2000), a second round of PCR was carried out with a primer that anneals specifically to *H. defensa* (Sandström et al., 2001) and further primers that check for DNA quality (Fukatsu et al., 2000, Russell and Moran, 2005); thermocycling conditions were similar to those described in the supplementary material of Leybourne et al. (2018), but with a shorter extension time. These routine checks cast some uncertainty on the proportion of aphids actually infected with *H. defensa* for the RB15/11 line. I have analysed my experiments on the assumption that *H. defensa* infection rates were complete or high for the individuals used in my experiment, and in the Discussion I explore the consequences of deviation from this assumption.

#### *4.2.3.1 Confirmatory genotyping of pea aphids using microsatellite markers*

Pea aphid DNA from three replicates of each clonal line, alongside a positive control line expected to be the same genotype as the experimental aphids and a negative control line expected to be of a different genotype, was extracted in NaOH. The DNA extraction protocol followed was adapted from Stanton et al. (1998). Individual aphids were incubated at 25°C in 20 µl 0.25 M NaOH for three hours. After further incubation in a thermocycler at 99°C for 3 min, 10 µl 0.25M HCl, 5µl 0.5M Tris-HCL (pH 8) and 5µl 2% Triton X-100 were added. The sample was incubated for a further 3 min at 99°C and left to cool to room temperature before storing at – 20°C. This DNA extraction method does not include a purification step in the process (such as phenol/chloroform washing). Superfluous cell material and non-purified extraction products are not removed from the extract and these can inhibit the PCR reaction. Therefore, to dilute the concentration of inhibiting factors, prior to amplification by PCR, the samples were thawed and diluted 1:10 with sterile water.

Aphid lines were genotyped based on length polymorphisms for microsatellite loci. Two published microsatellite markers (ApFO8M and ApHO5M with attached fluorophore 6-FAM) were selected to confirm the genotypes of the pea aphid lines (see Table 4.2 and Caillaud et al. [2004] for the 5’-3’ sequences of these primers). PCR was carried out using

**Table 4. 2:** Pea aphid microsatellite primer sequences, with 5'-3' sequences sourced from Caillaud et al. (2004).

| Locus | Primer name | Direction | 5'-3' primer sequence | Isolated from |
|---|---|---|---|---|
| **ApFO8M** | ApF08MF | Forward | TAATCCGTCGTAATTGCGTT | *Acyrthosiphon pisum* |
| | ApFO8MR | Reverse | TAAGCCCTCACTCACCCCTC | |
| **ApHO5M** | ApHO5MF | Forward | ACGAGAGCTTTCCGGCGTAT | *Acyrthosiphon pisum* |
| | ApHO5MR | Reverse | CAACGACGGCGGCTATACTA | |

**Table 4. 3:** Pea aphid microsatellite thermocycling conditions.

| Primer | Time | Temperature (°C) | Repeat | Action |
|---|---|---|---|---|
| **ApFO8M** | 3 min | 98 | - | Initial denaturation |
| | 30 s | 98 | 35 cycles | Denaturation |
| | 45 s | 60 | | Annealing |
| | 45 s | 72 | | Extension |
| | 5 min | 72 | - | Final extension |
| **ApHO5M** | 3 min | 98 | - | Initial denaturation |
| | 30 s | 98 | 35 cycles | Denaturation |
| | 45 s | 55 | | Annealing |
| | 45 s | 72 | | Extension |
| | 5 min | 72 | - | Final extension |

illustra$^{TM}$ puReTaq Ready-To-Go$^{TM}$ PCR Beads (GE Healthcare, UK). When these are used according to the manufacturer's instructions, a bead is reconstituted to a 25 µl volume. 22µl of sterile water, 1µl (20pmol/µl) of forward primer and 1µl (20pmol/µl) of reverse primer is added to each bead. A master mix is made for the total number of aphid samples tested to reduce pipetting errors.  Finally, 1µl of diluted DNA sample is added to each tube to make a final volume of 25µl. The final concentrations in the reaction are as follows: the concentration of each nucleotide is 200mM in 10 mM Tris-HCl, 50mM KCl and 1.5mM $MgCl_2$ and 2.5 units *Taq* DNA. However, these experimental procedures were found to be robust and reproducible, and so it was possible to reduce the PCR volume and maintain the quality of results. This was done by dividing the reaction mix from each bead into 8µl volumes and transferring to fresh sterile 0.2ml PCR tubes. 1 µl of the appropriate extracted diluted DNA sample was then added to each tube before the tubes were centrifuged and placed in the thermal cycler. PCR was conducted using a (Techne Prime) thermocycler (see Table 4.3 for thermocycling conditions for each primer), and products were stored at -20°C.

1.2µl of each of the PCR products was then mixed with 0.125µl of GeneScan™ Liz™ dye standard (ThermoFischer Scientific, UK) and suspended in 8.8µl Hi-Di™ Formamide (ThermoFischer, UK) in a non-skirted 96-well plate and sealed with an adhesive foil film. PCR products were sequenced by separating on an ABI 3730 DNA Analyser (Applied Biosystems, UK) and assessing product size using Peak Scanner Software v1.0 (Applied Biosystems, UK). The pea aphid clonal lines (GT218-cured and GT218-*H. defensa*) were confirmed to be of the same genotype as each other and as the positive control line (genotype 218) (Table 4.4).

**Table 4. 4:** Allele sizes (bp) recorded from the sequencing results of the four tested pea aphid lines (experimental lines GT218-cured and GT218-*H. defensa*, positive control line GT218-207, and negative control line LL01), with the two different primers. The differing peak sizes of the LL01 line genotype are emboldened and italicised.

| Primer | Line | Peak 1* | Peak 2* | Genotype | Role |
|---|---|---|---|---|---|
| **ApFO8M** | GT218-cured | 164.24 | 166.30 | 218 | Experimental |
| | GT218-*H. defensa* | 164.32 | 166.39 | 218 | Experimental |
| | GT218-207 | 164.90 | 167.01 | 218 | + control |
| | LL01 | 163.84 | ***175.36*** | LL01 | - control |
| **ApHO5M** | GT218-cured | 171.31 | 173.78 | 218 | Experimental |
| | GT218-*H. defensa* | 171.34 | 174.04 | 218 | Experimental |
| | GT218-207 | 171.12 | 173.59 | 218 | + control |
| | LL01 | ***174.11*** | ***176.76*** | LL01 | - control |

*****averaged peak sizes for the three replicates for each line

#### *4.2.3.2 Confirmatory facultative endosymbiont detection in pea aphids using diagnostic PCR screening*

Pea aphid samples were first flash-frozen in liquid nitrogen and homogenised using an ethanol-sterilised polypropylene micropestle. Following this, DNA was extracted from aphids using a DNeasy® Blood and Tissue kit (Qiagen, Crawley, UK), following the manufacturer's protocol. Extracted DNA was assessed using a Nanodrop ND-1000 (ThermoFischer Scientific, UK), and found to be of a suitable purity.

**Table 4. 5:** Primer names, targets, 5'-3' sequence and source for all primers used for facultative endosymbiont screening of pea aphid lines. Sourced from the supplementary material of Leybourne et al. (2018).

| Primer | Target | Sequence (5'–3') | Primer Source |
|---|---|---|---|
| **16SA1** | 16S rDNA<br>(positive for bacterial presence/aphid primary endosymbiont) | AGAGGTTGATCMTGGCTCAG | Fukatsu and Nikoh, 2000 |
| **16SB1** | | TACGGYTACCTTGTTACGACTT | |
| **10F** | 16-23S rDNA including Intergenic Spacer Region<br>(positive for secondary endosymbionts) | AGTTTGATCATGGCTCAGATTG | Sandström et al., 2001 |
| **480R** | | CACGGTACTGGTTCACTATCGGTC | |
| **16SA1** | *Regiella insecticola* 16S rDNA | AGAGGTTGATCMTGGCTCAG | Fukatsu and Nikoh, 2000 |
| **PASScmp** | | GCAATGTCTTATTAACACAT | Fukatsu et al., 2000 |
| **PABSF** | *Hamiltonella defensa* 16S rDNA | AGCGCAGTTTACTGAGTTCA | Darby and Douglas, 2003 |
| **16SB1** | | TACGGYTACCTTGTTACGACTT | Fukatsu and Nikoh, 2000 |
| **U99F** | *Serratia symbiotica* 16S rDNA | ATCGGGGAGTAGCTTGCTAC | Sandström et al., 2001 |
| **16SB1** | | TACGGYTACCTTGTTACGACTT | Fukatsu and Nikoh, 2000 |

**Table 4.5 (continued)**

| | | | |
|---|---|---|---|
| **PAXSF** | *PAXS*<br>16S rDNA | GAAGCAATGCAAAGAGTGTTGC | Guay et al., 2009 |
| **1507R** | | TACCTTGTTACGACTTCACCCCAG | Sandström et al., 2001 |
| **16SA1** | *Rickettsia sp.*<br>16S rDNA | AGAGGTTGATCMTGGCTCAG | Fukatsu and Nikoh, 2000 |
| **Rick16SR** | | CATCCATCAGCGATAAATCTTTC | Fukatsu et al., 2000 |
| **16SA1** | *Spiroplasma sp.*<br>16S rDNA | AGAGGTTGATCMTGGCTCAG | Fukatsu and Nikoh, 2000 |
| **TKSSsp** | | TAGCCGTGGCTTTCTGGTAA | |
| **RCL16-211F** | *Rickettsiella sp.*<br>16S rDNA | GGGCCTTGCGCTCTAGGT | Tsuchida et al., 2010 |
| **RCL16S-470R** | | TGGGTACCGTCACAGTAATCGA | |

A diagnostic PCR screen was conducted, targeting the universal eubacterial 16S rDNA gene, the 16-23S rDNA region (including intergenic spacer region), and the specific 16S rDNA target sequence of the seven most frequently detected aphid endosymbionts: *Regiella insecticola*, *Serratia symbiotica*, PAXS, *Spiroplasma* sp., *Rickettsia* sp., *Rickettsiella* sp. and, key to this study, *Hamiltonella defensa* (see Table 4.5). The universal eubacterial 16S rDNA was amplified in order to confirm the presence of bacterial DNA in aphid samples (i.e. the primary, obligate symbiont *Buchnera*). The 16-23S rDNA was targeted to determine the presence or absence of facultative (or secondary) endosymbionts; if a PCR product was detected, the symbiont-specific 16S rDNA sequences were amplified to identify which facultative symbiont(s) were present. The reactions were conducted using a ProFlex thermocycler (Applied Biosystems, UK) in a final reaction volume of 25 µl, with reaction concentrations of 1.5 mM MgCl2, 250 µM of mixed dNTP's, 1 µM forward primer, 1 µM reverse primer, 1× Green GoTaq® reaction buffer (Promega, UK) and 1.25 U GoTaq® DNA Polymerase (Promega, UK), and with 1.1µl of extracted DNA. The final reaction mixtures were centrifuged to mix before reactions were then conducted in a ProFlex PCR System (Applied Biosystems UK) thermocycler (see Table 4.6).

An aliquot (8 µl) of the amplified product, alongside a 100bp DNA ladder, was separated and visualised by electrophoresis on 1% agarose gels using SYBR Safe® DNA staining agent. The presence or absence of endosymbionts was determined by comparison of PCR product sizes with positive and negative controls for each target sequence using DNA size markers. The GT218-*H defensa* line tested positive for *H. defensa* while the GT218-cured line was negative for *H. defensa*. Neither experimental line was found to possess any other facultative endosymbiont (Table 4.1).

**Table 4. 6:** Thermocycling conditions for diagnostic PCR of pea aphid facultative endosymbionts. Sourced from the supplementary material of Leybourne et al. (2018).

| Target | Time | Temp. (°C) | Repeat | Action |
|---|---|---|---|---|
| **16S rDNA** | 5 minutes | 95 | - | Initial denaturation |
| | 30 s | 95 | | Denaturation |
| | 45 s | 60 | 35 cycles | Annealing |
| | 45 s | 72 | | Extension |
| | 7 minutes | 72 | - | Final extension |
| **16S rDNA; *H. defensa.*, *R. insecticola.*, *S. symbiotica.* 16-23S rDNA** | 2 minutes | 95 | - | Initial denaturation |
| | 30 s | 95 | | Denaturation |
| | 30 s | 55 | 35 cycles | Annealing |
| | 3 minutes | 72 | | Extension |
| | 7 minutes | 72 | - | Final extension |
| **16S rDNA; *PAXS, Rickettsiella sp.*** | 2 minutes | 95 | - | Initial denaturation |
| | 30 s | 95 | | Denaturation |
| | 30 s | 55 | 35 cycles | Annealing |
| | 90 s | 72 | | Extension |
| | 5 minutes | 72 | - | Final extension |
| **16S rDNA *Rickettsia sp.*, Spiroplasma sp.** | 2 minutes | 95 | - | Initial denaturation |
| | 60 s | 95 | | Denaturation |
| | 60 s | 55 | 35 cycles | Annealing |
| | 2 minutes | 72 | | Extension |
| | 5 minutes | 72 | - | Final extension |

### 4.2.4 Experimental protocol

Assays were conducted in a glasshouse with lighting conditions 16:8 h light:dark and 20:14°C. The experimental set-up was positioned away from ventilation fans to minimise disturbance. For each assay, a plant pot (15cm diameter) with two young plants was encased by a mesh net supported by a metal frame. Plants were trimmed minimally if needed to prevent contact between plant material and the mesh net. Pots were then infested in the afternoon with 16 wingless immature aphids and left undisturbed overnight. Ten minutes before each assay, the pot was placed into a plastic tray (6 cm H x 60 cm W x 39 cm D) and the frame and mesh net removed. A JVC Everio HDD GZ-MG330AE hard disk camcorder fixed to an Ex-Pro® TR-654 professional photographic camera tripod (135 cm H x 49 cm W) positioned beside the tray was angled to give a planar view of the pot.

Throughout trials, I wore a surgical mask to prevent plants or insects from being disturbed by air movement. All insects were transferred from cups to plants and vice versa using a fine artist's paintbrush. Each assay started at either 0900, 1130, 1400, or 1630hrs. When filming began, the number of aphids remaining on plants was recorded using a Silverline 129472 telescopic inspection mirror (Somerset, UK) to determine if any aphids had escaped overnight and/or fallen onto the soil. A predator (ladybird adult or lacewing larvae) was then added to the base of a randomly-selected plant to start each assay.

Once each assay had started, the predator was observed for a 30-minute period, during which its movements and aphid behaviours deemed to be in response to the predator or its movements on the predator's nearby plant sections (considered as stem, petiole, petiolule, leaf upperside or leaf underside) were dictated to the camera. Aphid behaviours were classified as: 'drop', 'walk' 'kick', 'shift', and 'no' response. 'Drop' was where an aphid dropped from its current plant section, usually to the substrate below. 'Walk' included behaviours ranging from backing-up a pace or two to running quickly away; drawing a distinct line between just backing-up and running to escape would have been difficult as often a back-up could transition to a walk and then a run, so all were classed as variations of 'walking' behaviour. 'Kick' described the quick motion/flicking of a leg or two legs in the direction of the predator. 'Shift' was defined as when aphids moved their body or antennae in response to the stimulus but without stepping anywhere; this category is not typically recorded in the literature, but it was deemed useful to differentiate between some slight motion/acknowledgement of predator presence and no response. ‘No’ was where the aphid remained motionless despite the predator having made contact.

If aphids departed from the pot by walking during the 30-minute observation period, they were not replaced. If an aphid dropped off the side of the pot (this happened mainly with the potato aphids on the larger plants), it was replaced on the substrate at the edge of the pot. If a predator climbed the rim of the pot, it was moved to the base of the plant it had visited least recently (or not at all). If the predator did not encounter an aphid for five minutes it was also moved to the base of the least recent (or unexplored) plant. If a predator successfully captured an aphid, it was given 10 minutes to finish consuming its prey and continue moving otherwise it was replaced entirely with a predator of the same species. If a predator was motionless for two uninterrupted minutes or (ladybird-specific) flew away, it was also replaced with a fresh predator of the same species. After any occasion where a predator needed to be replaced, the next predator was introduced to the least recently-explored (or unexplored) plant. The timing of the observation period was paused while predators were being replaced, and restarted when the new predator was on a plant. Each predator was used only once. The observation period continued until 30 minutes had passed; the final predator, on some occasions still the initial predator, was then removed from the pot.

A total of 16 pea aphid trials took place in May 2019. Each of the two lines were tested across eight trials, giving a total of 16 trials, and the predator type used in each trial was randomised. A total of 32 potato aphid trials took place in July 2019. Each of the four aphid lines were tested across eight assays, giving a total of 32 assays, with predator type assigned at random to the order of the assays. In the potato aphid trials, it was ensured that each line experienced each predator type in 50% of the trials, but the order in which trials with the different predator types were conducted was still randomised. Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba, 2016) was used to aid data collection from the video footage. Using the dictations of all predator movements and aphid responses on the predator’s current plant section at any given moment in time, the antipredator behaviours in response to the predator’s presence were coded.

### 4.2.5 Analysis

Across both aphid species, evasive defences made up the majority of recorded responses, but aggressive defences were the second most frequent response type. For this reason, the dependent variables analysed for this study were: the total number of defensive responses to predators per trial, the total number of evasive defensive responses (dropping or backing-up/walking away) per trial, the total number of aggressive defensive responses (kicking) per trial, and the proportion of the overall behavioural responses evasive defences represented per trial. In this chapter, my focus is on the influence of various internal and external factors on defence overall, and so the evasive responses of dropping and backing-up/walking away were grouped together in order to avoid the models spending lots of degrees of freedom on the minutiae of each different defence; I consider the fine detail of the use of different defences in response to different predator types in Chapter Five. Generalised linear mixed models (GLMMs) were fitted using the lme4 package (Bates et al., 2015) in R version 3.6.3 (R Core Team, 2020), assuming either a Poisson (for count data) or binomial (for proportions) error, with log- or logit-link functions respectively. For the pea aphid trials, the effect of aphid symbiont status (the two lines tested differed in presence or absence of *H. defensa*) and predator type (lacewing larva or ladybird adult) on each dependent variable was tested, with time of day and date considered as potential random factors in the statistical modelling. For the potato aphid trials, the effect of aphid symbiont status, aphid genotype (parasitism-resistant or -susceptible), and predator type on each dependent variable was tested. Again, time and date were considered as random factors.

For each dependent variable, a suite of models was produced. Each model contained all of the essential fixed effects detailed above, but varied in: i) their inclusion of either one or both of the random factors as random intercepts (all models included at least one of the random effects), ii) their inclusion or exclusion of the possible interactions between the essential fixed effect terms. Models for each possible combination of random effect inclusions and interaction inclusions/exclusions were produced and compared using the Akaike information criterion (AIC), where the best fitting model produces the lowest AIC score, but models that produce scores within 2 are considered equally likely. Best models were often considered to be the model that produced the lowest AIC value. However, where a model that produced the absolute lowest AIC value included non-significant interaction terms, in turn the next lowest AIC-producing models were checked and selected as best if they contained either a significant interaction term or only the essential fixed effect terms and random effects. Where a model that included one, but not both, random effects was initially deemed the best, if the equivalent version of the model but with both random effects was within an AIC score of 2 of the original best model, the version with both random effects was instead considered as the final best model; exceptions to this included instances where the model with both random effects produced over-fitting warnings when modelled while the original best model did not, and cases where the model with both random effects indicated that any significant interactions from the original best model were no longer significant.

Checks to confirm selection of the final best model were also informed by: point estimates and 95% confidence intervals, visualisations of the linear prediction of possible interactions, and descriptive statistics of the dependent variable’s original data. The significance of all essential fixed effect terms and any interactions, in the final best models were extracted from model summaries. Additionally, likelihood ratio tests were used to confirm the significance of interaction terms and any individual essential terms not involved in interactions. All of the final models were then checked using the DHARMa package function testResiduals (Hartig, 2020) which supported the models, finding no issues with the distribution of residuals, outliers, or dispersion of data.

## 4.3 Results

During the 16 pea aphid trials, the random assignment of predator types resulted in six of the eight trials testing aphids of the GT218 (cured) line utilising ladybird adults, and the remaining two trials utilising lacewing larvae. Correspondingly, six of the eight trials testing the GT218 (*H. defensa*) line utilised lacewing larvae, while the other two utilised ladybird adults. Pea aphids from both lines were observed to exhibit dropping, walking, kicking, and shifting behaviours in response to both predator types, but none of the pea aphids tested remained motionless (i.e. showed the ‘no’ response) following contact with a predator. Throughout trials, only one pea aphid was captured and consumed by a predator. The aphid was from the GT218 (cured) line, and was predated by a ladybird adult after an attempted drop escape landed it only millimetres away onto a petiole directly below.

As mentioned in section 4.2.4, during the 32 potato aphid trials it was ensured that predator type was assigned equally across the eight trials for each of the four aphid lines tested, although in a random order, so that four of the trials with each line utilised ladybird adults and the other four utilised lacewing larvae. All four potato aphid lines were observed to exhibit dropping, walking, kicking, and shifting behaviours in response to both predator types. The response to remain motionless was also seen across the potato paid lines during encounters with both predator types, with the exception of the parasitism-resistant (genotype 1) *H. defensa*-infected line RB15/11; one individual of this line remained motionless in response to contact from a lacewing larvae, but there were no cases where aphids of this line remained motionless in response to contact from a ladybird adult. During trials, seven potato aphids with the parasitism-resistant genotype were captured and consumed by predators, three of which did not harbour *H. defensa* (line MW16/67) and four of which did (line RB15/11). Additionally, ten potato aphids belonging to the parasitism-susceptible genotype were captured by predators, five of which were infected with *H. defensa* (line AK14/01) and five of which were not (line MW16/48). Aphids from each line were captured by both predator types, and there was at least one example from each line where an aphid was grabbed ahead of showing any response to the predator.

The results below are structured to address the three main research questions, with details of specific models given in tables.

### 4.3.1 Can the effects of *H. defensa* infection on antipredator defence be observed in pea aphids faced with predators on live plants?

Whether pea aphids were infected with *H. defensa* or not appeared to have a significant effect ($P < 0.001$, z-value = 5.24) on the total count of behavioural responses to predators recorded per trial, with the infected GT218 (*H. defensa*) line exhibiting a higher overall mean count than the uninfected GT218 (cured) line (Figure 4.1a). Considering evasive responses alone (dropping and walking away/backing-up), symbiont status did not appear to significantly influence the total counts of defences (Figure 4.1b, P = 0.867, z-value = -0.17, LRT P = 0.868, LRT $\chi^2_{(-1)} = 0.03$), but did significantly influence the proportion of evasive defences used by the two different aphid lines (Figure 4.1d, $P < 0.001$, z-value = -3.39, LRT $P < 0.001$, LRT $\chi^2_{(-1)} = 10.85$). The GT218 (cured) line had a slightly higher mean count of total evasive defences, and utilised proportionately more evasive defences, compared to the GT218 (*H. defensa*) line. Symbiont status was also found to have a significant influence on the total counts of aggressive defence (kicking) in pea aphids ($P < 0.001$, z-value = 5.59), though here with the GT218 (*H. defensa*) line exhibiting a higher mean count than the GT218 (cured) line (Figure 4.1c).

### 4.3.2 Does symbiont infection differentially affect pea aphid behaviour against different predator types?

The predator type pea aphids were faced with had a significant influence on the total counts of all responses recorded (P <0.001, z-value = 5.66), with ladybird adults provoking significantly more responses than lacewing larvae (Figure 1a). Ladybird adults were also found to provoke significantly higher total counts of evasive responses (Figure 4.1b, P <0.001, z-value = 4.67, LRT P <0.001, LRT $\chi^2_{(-1)} = 23.16$) and aggressive responses (Figure 4.1c, P <0.001, z-value = 4.12), as well as proportionately more evasive responses (Figure 4.1d, P = 0.043, z-value = 2.02, LRT P = 0.042, LRT $\chi^2_{(-1)} = 4.14$).

An interaction between symbiont status and predator type was found to be a significant predictor of both the total counts of all responses (Figure 4.1a, P <0.001, z-value = -9.00, LRT P <0.001, LRT $\chi^2_{(-1)} = 30.47$) and the total counts of aggressive responses (Figure 4.1c, P <0.001, z-value = -7.84, LRT P <0.001, LRT $\chi^2_{(-1)} = 35.38$). Both predator types, though, elicited slightly higher mean counts of total defences (Figure 4.1a) and aggressive responses (Figure 4.1c) in the GT218 (*H. defensa*) line compared to the GT218 (cured) line. Ladybird adults and lacewing larvae were also both met with a higher proportion of evasive responses in the repertoire of the GT218 (cured) line compared to the GT218 (*H. defensa*) line (Figure 4.1d).

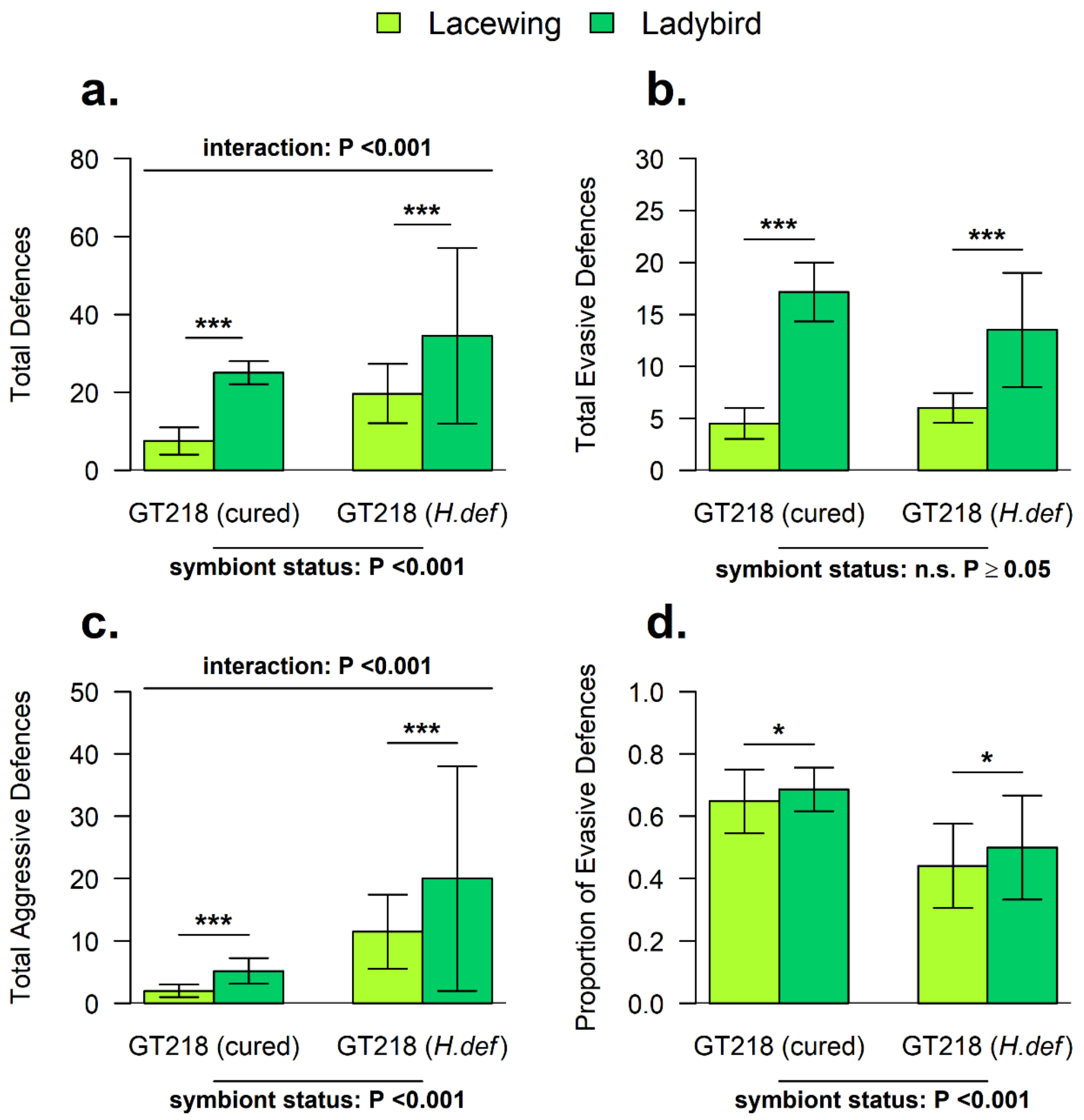

**Figure 4. 1:** Effect of *H. defensa* infection status (cured or infected), predator type (lacewing larva or ladybird adult), and potential interaction between the two, on the behavioural responses of pea aphids. Panels show the means (±SE) per trial of: **a)** the total counts of defences, **b)** the total counts of evasive defences (dropping or backing-up/walking away), **c)** the total counts of aggressive defences (kicking), and **d)** the proportion of evasive defences (dropping or backing-up/walking away) relative to the total defences recorded. Horizontal lines describing significance correspond to the fixed predictor variables included in the final GLMM models for each dependent variable; see Tables 4.7 and 4.8 for further details of these and the random effects included. Statistical significance was evaluated from those final models, with the significance of stimulus shown as: *** P <0.001 and * P <0.05. More trials with GT218 (cured) aphids recorded responses to ladybird adults (n = 6) than lacewing larvae (n = 2), while more trials with GT218 (*H. defensa*) aphids recorded responses to lacewing larvae (n = 6) than ladybird adults (n = 2).

**Table 4. 7:** Summary table outputs from the best fit pea aphid GLMMs for the individual fixed effect terms included in all models: symbiont status (presence or absence of *H. defensa*) and stimulus (predator type). Symbiont status was coded with the cured aphid line as the baseline, such that a positive estimate value suggests that the line infected with *H. defensa* exhibited a greater mean frequency or proportion of behaviour (depending on the dependent variable concerned). Stimulus was coded with lacewing larvae predators as the baseline, such that a positive estimate value suggests that the ladybird adult predators provoked a greater mean frequency or proportion of behaviour (depending on the dependent variable concerned). Also shown for each dependent variable is the random effect(s) included in the best fit model and the estimate value for the intercept. Significant p-values are emboldened.

| | Random effects | | Intercept | Symbiont Status | | | | Stimulus | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dependent variable** | **Time** | **Date** | **Estimate** | **Estimate** | **Std. Error** | **z-value** | **p-value** | **Estimate** | **Std. Error** | **z-value** | **p-value** |
| **Total defensive** | YES | YES | 1.480 | 1.949 | 0.372 | 5.238 | **<0.001** | 2.041 | 0.361 | 5.659 | **<0.001** |
| **Total evasive** | YES | YES | 1.745 | -0.035 | 0.206 | -0.168 | 0.867 | 1.052 | 0.225 | 4.671 | **<0.001** |
| **Total aggressive** | YES | YES | -0.949 | 4.287 | 0.767 | 5.592 | **<0.001** | 3.036 | 0.736 | 4.122 | **<0.001** |
| **Proportion evasive** | NO | YES | 0.225 | -1.049 | 0.310 | -3.390 | **<0.001** | 0.651 | 0.322 | 2.022 | **0.043** |

**Table 4. 8 (right):** Summary table outputs from the best fit pea aphid GLMMs for the interaction term included in some models: the interaction between symbiont status (presence or absence of *H. defensa*) and stimulus (lacewing larva or ladybird adult). Where the best fit GLMM did not include this interaction term, N/A is shown. Significant p-values are emboldened.

| | Symbiont Status*Stimulus | | | |
|---|---|---|---|---|
| **Dependent variable** | **Estimate** | **Std. Error** | **z-value** | **p-value** |
| **Total defensive** | -3.378 | 0.668 | -5.060 | **<0.001** |
| **Total evasive** | N/A | N/A | N/A | N/A |
| **Total aggressive** | -6.664 | 1.352 | -4.928 | **<0.001** |
| **Proportion evasive** | N/A | N/A | N/A | N/A |

### 4.3.3 Is the effect due to symbiont presence per se or due to parasitism resistance generally?

Infection with *H. defensa* (z-value = 6.31), aphid genotype (z-value = -7.26, LRT $\chi^2_{(-1)}$ = 53.68), predator type (z-value = 9.29), and the interaction between symbiont status and predator type (z-value = -9.00, LRT $\chi^2_{(-1)}$ = 78.37) were all suggested to be significant predictors (P <0.001) of the total counts of all responses potato aphids exhibited (Figure 4.2a). Across both parasitism-resistant (genotype 1) and parasitism-susceptible (genotype 2) genotypes, the lines infected with *H. defensa* (RB15/11 and AK14/01) appeared to show greater differentiation than the uninfected lines (MW16/67 and MW16/48) in the number of responses they exhibited towards the two predator types, with ladybird adults eliciting notably higher mean counts of responses than lacewing larvae (Figure 4.2a). Aphids from parasitism-resistant genotype lines, though, also appeared to exhibit slightly higher total mean counts of responses than their symbiont-status-equivalent susceptible lines when faced with the same predator type (Figure 4.2a).

These trends also held when the total counts of only evasive defences (dropping or backing-up/walking away) were considered (Figure 4.2b). Again, the significant predictor variables consisted of symbiont status (P = 0.022, z-value = 2.29), aphid genotype (P <0.001, z-value = -5.44, LRT P <0.001, LRT $\chi^2_{(-1)}$ = 30.65), predator type (P <0.001, z-value = 5.89), and the interaction between symbiont status and predator type (P <0.001, z-value = -4.05, LRT P <0.001, LRT $\chi^2_{(-1)}$ = 17.59). The lines not infected with *H. defensa* showed significantly higher mean counts of evasive defences in response to ladybird adults than they did in response to lacewing larvae (Figure 4.2b). However, looking at the proportion of the different lines' behavioural responses that constituted evasive defences (Figure 4.2d), symbiont status did not have a significant effect (P = 0.287, z-value = -1.07, LRT P = 0.29, LRT $\chi^2_{(-1)}$ = 1.13). Predator type (P <0.001, z-value = 3.36) and aphid genotype (P = 0.045, z-value = 2.01) were both found to be significant predictors of the proportion of evasive defences utilised, though, as was the interaction between stimulus and genotype (P = 0.034, z-value = -2.12, LRT P = 0.03, LRT $\chi^2_{(-1)}$ = 4.52). The parasitism-susceptible genotype lines may have exhibited a slightly greater differentiation in the proportion of evasive responses used against the different predator types, but across all lines the predator type was clearly the most important predictor; all lines used proportionately more evasive defences in response to ladybird adults compared to lacewing larvae (Figure 4.2d).

Considering the total counts of aggressive defences (kicking), infection with *H. defensa* ($P < 0.001$, z-value = 6.24), aphid genotype ($P < 0.001$, z-value = -4.53, LRT $P < 0.001$, LRT $\chi^2_{(-1)} = 22.33$), predator type ($P < 0.001$, z-value = 6.83), and the interaction between symbiont status and predator type ($P < 0.001$, z-value = -7.84, LRT $P < 0.001$, LRT $\chi^2_{(-1)} = 78.00$) were all found to be significant predictors (Figure 4.2c). Both parasitism-resistant genotype lines appeared to differentiate the number of aggressive responses dependent on predator type, but with the line negative for *H. defensa* (MW16/67) tending to exhibit more kicks towards lacewing larvae than ladybird adults and the line harbouring *H. defensa* (RB15/11) exhibiting significantly more kicks towards ladybird adults than lacewing larvae. The *H. defensa*-positive parasitism-susceptible genotype line (AK14/01) also appeared to differentiate the number of aggressive defences used against different predator types, kicking more against ladybird adults than lacewing larvae, but the *H. defensa*-negative parasitism-susceptible genotype line did not show any significant stimulus differentiation (Figure 4.2c).

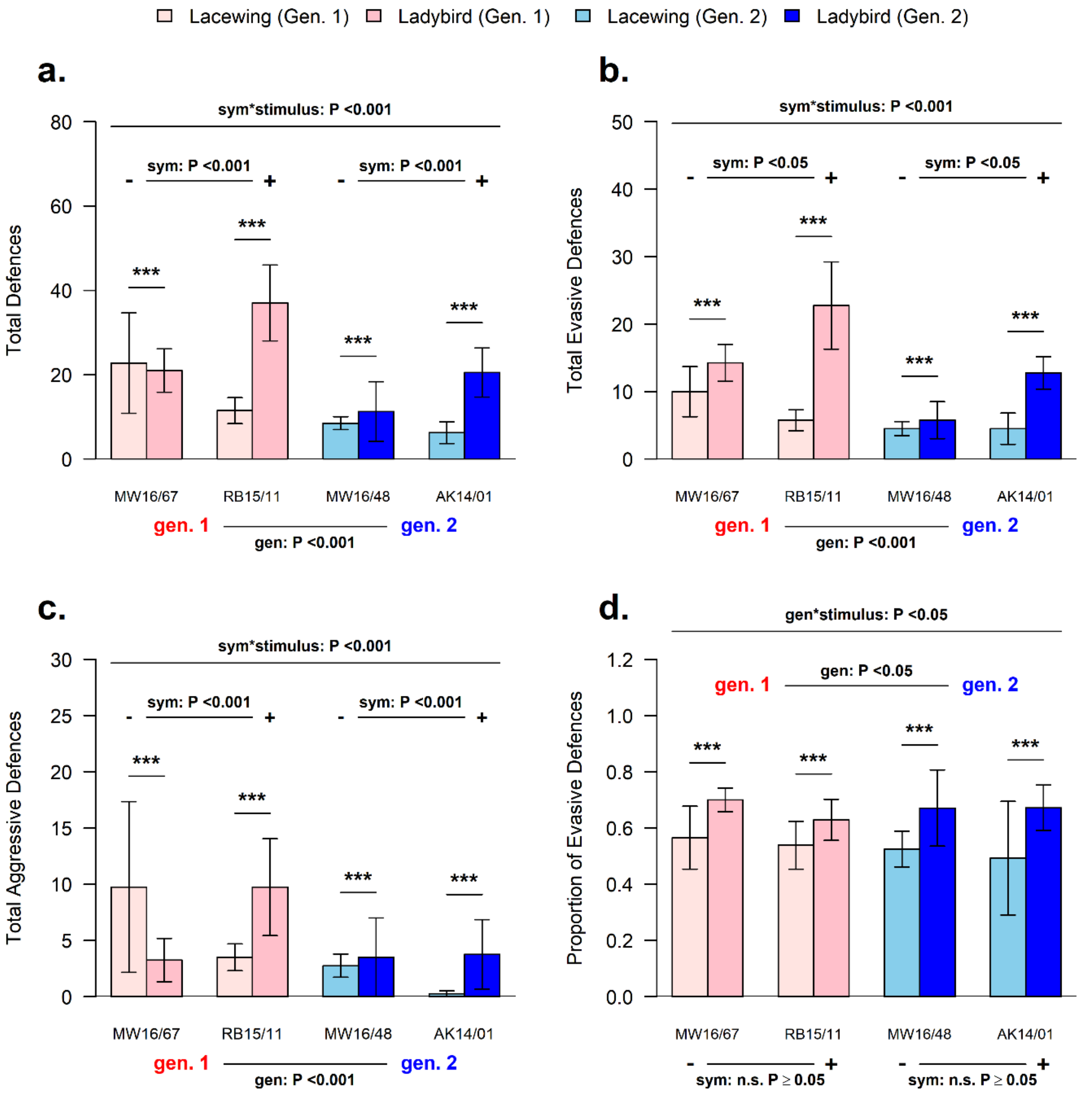

**Figure 4. 2:** Effect of *H. defensa* infection status (‘sym’, - uninfected or + infected), genotype (‘gen’, 1 parasitism-resistant or 2 parasitism-susceptible), predator type (‘stimulus’, lacewing larva or ladybird adult), and potential interactions between these, on the behavioural responses of potato aphids. Panels show the means (±SE) per trial of: **a)** the total counts of defences, **b)** the total counts of evasive defences (dropping or backing-up/walking away) , **c)** the total counts of aggressive defences (kicking) , and **d)** the proportion of evasive defences (dropping or backing-up/walking away) relative to the total defences recorded. Horizontal lines describing significance correspond to the fixed predictor variables included in the final GLMM models for each dependent variable; see Tables 4.9 and 4.10 for further details of these and the random effects included. Statistical significance was evaluated from those final models, with the significance of stimulus shown as: *** P <0.001. All four potato aphid lines experienced an equal number of trials with lacewing larvae (n = 4) and ladybird adults (n = 4).

**Table 4. 9:** Summary table outputs from the best fit potato aphid GLMM for the individual fixed effect terms included in all models. For each of the following fixed effects, the first listed level of the term was coded as the baseline, with a positive estimate value indicating a relatively greater mean frequency or proportion of behaviour (depending on the dependent variable concerned) associated with the second listed level of the term: symbiont status (presence/absence of *H. defensa*), genotype (1: parasitism-resistant or 2: -susceptible) and stimulus (lacewing larva or ladybird adult). Also shown for each dependent variable is the random effect(s) included in the best fit model and the estimate value for the intercept. Significant p-values are emboldened.

| | **Random effects** | | **Intercept** | **Symbiont Status** | | | | **Genotype** | | | | **Stimulus** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dependent variable** | **Time** | **Date** | **Estimate** | **Estimate** | **Std. Error** | **z-value** | **p-value** | **Estimate** | **Std. Error** | **z-value** | **p-value** | **Estimate** | **Std. Error** | **z-value** | **p-value** |
| **Total defensive** | YES | YES | 2.023 | 1.200 | 0.190 | 6.314 | **<0.001** | -0.686 | 0.094 | -7.262 | **<0.001** | 1.899 | 0.204 | 9.293 | **<0.001** |
| **Total evasive** | YES | YES | 1.758 | 0.549 | 0.239 | 2.293 | **0.022** | -0.652 | 0.120 | -5.436 | **<0.001** | 1.443 | 0.245 | 5.891 | **<0.001** |
| **Total aggressive** | YES | YES | -0.182 | 2.503 | 0.401 | 6.235 | **<0.001** | -0.989 | 0.218 | -4.531 | **<0.001** | 3.088 | 0.452 | 6.826 | **<0.001** |
| **Proportion evasive** | YES | NO | -0.151 | -0.202 | 0.190 | -1.065 | 0.287 | 0.666 | 0.332 | 2.005 | **0.045** | 0.913 | 0.272 | 3.358 | **<0.001** |

**Table 4. 10:** Summary table outputs from the best fit potato aphid GLMM for the interaction terms included in some models: the interaction between symbiont status (presence/absence of *H. defensa*) and stimulus (lacewing larvae or ladybird adult), and the interaction between genotype (1: parasitism-resistant or 2: -susceptible) and stimulus. Where the best fit GLMM did not include the interaction term, N/A is shown. Significant p-values are emboldened.

| | **Symbiont Status*Stimulus** | | | | **Genotype*Stimulus** | | | |
|---|---|---|---|---|---|---|---|---|
| **Dependent variable** | **Estimate** | **Std. Error** | **z-value** | **p-value** | **Estimate** | **Std. Error** | **z-value** | **p-value** |
| **Total defensive** | -2.413 | 0.268 | -9.004 | **<0.001** | N/A | N/A | N/A | N/A |
| **Total evasive** | -1.384 | 0.342 | -4.048 | **<0.001** | N/A | N/A | N/A | N/A |
| **Total aggressive** | -4.776 | 0.609 | -7.839 | **<0.001** | N/A | N/A | N/A | N/A |
| **Proportion evasive** | N/A | N/A | N/A | N/A | -0.844 | 0.398 | -2.119 | **0.034** |

## 4.4 Discussion

The antipredator behaviours prey exhibit during encounters with their natural enemies have a strong bearing on their immediate survival likelihood and subsequent fitness. This study investigated the role of the symbiont *H. defensa* on the behavioural portfolios of pea aphids and potato aphids when faced with different predatory threats. The two pea aphid lines studied were both of the same natural genotype and were both reared under the same conditions. The only difference between them was that they differed in their *H. defensa* infection status; one was naturally infected and the other had been experimentally cured of the endosymbiont. Where possible, it is preferable for aphids' genetic backgrounds and infection statuses to originate from individuals sampled in nature in order to avoid artefactual and detrimental effects of selective elimination and transfection (Polin et al., 2014). However, limited by the availability of pea aphid lines known to be of the same genotype, I selected to test a genotype-matching line from which naturally-occurring *H. defensa* had been eliminated rather than test lines of a genotype with *H. defensa* naturally absent where one had been artificially infected with the symbiont. This was in order to avoid the risk of incompatibilities that can occur between the host and introduced symbiont genotypes following transfection manipulations (Polin et al., 2014). This also enabled me to compare the lines directly, in the knowledge that any apparent differences in behavioural responses to predators could be attributed to *H. defensa* infection.

Pea aphids infected with *H. defensa* were found to exhibit proportionately fewer evasive behaviours (dropping and walking away) than cured aphids when faced with predators on live plants. This supports the finding of Polin et al. (2014) that pea aphids – for whom *H. defensa* infection can provide parasitism resistance (Oliver et al., 2003, Oliver et al., 2006, Guay et al., 2009, Donald et al., 2016) – exhibit fewer evasive behaviours in the presence of foraging ladybird predators. However, pea aphids infected with *H. defensa* were also seen to exhibit significantly greater mean frequencies of all recorded defences and, specifically, aggressive kicking defence in response to ladybirds than did cured pea aphids. This contrasts with previous findings of *H. defensa*-infected aphids exhibiting relatively fewer aggressive defences towards natural enemies (Dion et al., 2011, Polin et al., 2014). While *H. defensa*, or the parasitism-resistance associated with it in pea aphids (Oliver et al., 2003, Oliver et al., 2006, Guay et al., 2009, Donald et al., 2016), may reduce aphids' propensity to utilise particular antipredator behaviours, perhaps it does not cause an outright reduction in defence.

Pea aphids harbouring *H. defensa* here showed a tendency to utilise fewer energetically-costly defences relative to cured pea aphids, but still exhibited many defensive responses, including a greater tendency towards aggressive kicking. This finding could result from the more naturalistic set-up on live plants compared to the excised leaves of previous work (Dion et al., 2011, Polin et al., 2014), as well as the consideration of 'shifting' behaviour beyond evasive and aggressive activity alone. Future studies also considering a broader array of defensive behaviour categories may further help to draw a distinction between an overall reduction in defensiveness and a reduced tendency to utilise particular defences in favour of others.

Alongside symbiont infection status, the influence of predator type on pea aphid defensive behaviour was explored. Different predators are known to utilise different foraging styles, which can consequently provoke different behavioural responses from their prey. For example, syrphid larvae have been reported as foraging relatively slowly and provoking primarily back-up responses from pea aphids, while coccinellid beetles forage more vigorously causing greater plant vibrations and triggering more dropping escape by pea aphids (Brodsky and Barlow, 1986). In my study, predator type appeared to be the key predictor of pea aphid defence frequencies (more so than symbiont infection status), with ladybird adults provoking significantly more evasive, aggressive, and total counts of behaviours than lacewing larvae. Ladybirds were observed to be much faster-moving and appeared to actively forage more than lacewing larvae, although the foraging style was not directly quantified. For pea aphids, then, perhaps utilising a greater frequency of defensive behaviours (including dropping as the most energetically-costly option) is necessary in order to avoid predation by more active predators, such as ladybird adults. As slower-foraging predators, the lacewing larvae may: cause fewer plant vibrations, triggering fewer defences; be less thorough in their search and therefore evaded by minimising visual cues like movement; and/or be deterred more easily by fewer or less costly aphid defences. The influence of predator type on the use of different aphid defences and the timing of dropping behaviour, in particular, in relation to contact with a predator are explored further in Chapter Five.

Interestingly, even though pea aphids infected with *H. defensa* demonstrated a weaker tendency to use evasive responses overall, and particularly in response to lacewing larvae, they did not consequently experience a greater rate of predation. While Polin et al. (2014)

found that infected pea aphids suffered from higher predation than symbiont-free aphids – a finding attributed to infected aphids exhibiting reduced defences – symbiont infection did not affect susceptibility to predation in this study. Only one pea aphid was consumed during assays (by a ladybird after an attempted drop escape) and this individual was from the cured line. None of the pea aphids of either line failed to respond to contact with a predator. Although encounter rates between predator and prey were not quantified in this study there is no reason to assume that predators explored plants to different degrees depending on the aphid line, and so the presence of *H. defensa* in pea aphids did not seem to make this line more likely to be predated.

It is important to bear in mind that different strains of *H. defensa* can vary in the strength of protection conferred to pea aphids against parasitoids (Chevignon et al., 2018). If resistance to parasitoids and behaviour are linked, reduced protection against parasitism could mean that the frequency of defensive behaviours exhibited by infected hosts (towards parasitoids or predators or both) should, in turn, be expected to be less reduced. Protection from parasitism has been found to depend on the strain of *H. defensa* and the symbiotic consortium of the host (Leclair et al., 2016), as well as the identity of the attacking parasitoid species, and the pea aphid biotype concerned (McLean and Godfray, 2015). That is to say that even in pea aphids it is possible to find strains that provide little or no protection from parasitism to their aphid hosts (McLean and Godfray, 2015, Leclair et al., 2016). As found for the black bean aphid (Hafer-Hahmann and Vorburger, 2020), diversity in the strains of *H. defensa* in pea aphids may be driven by the diversity – or lack of diversity – in the parasitoid species they encounter, and so the specificity or generality of protection in pea aphids may depend in part on their coevolutionary history with their natural enemies. The relationship between aphid infection with *H. defensa* strains that provide varying levels of protection from parasitism, or perhaps parasitoid-specific protection, and the resulting effects on aphid defensive behaviours in response to their predators (as well as their parasitoids) is certainly worthy of further investigation in future studies that also consider the relative effectiveness of different defences against different predator types (and the consequences for both prey and predator populations). *H. defensa* protection from parasitism can also fail at higher temperatures (Bensadia et al., 2006), although some pea aphid genotype and *H. defensa* strain combinations are more robust than others (Doremus et al., 2018).

Beyond mutualistic relationships between pea aphids and *H. defensa,* Łukasik et al. (2013) found that infection with *H. defensa* did not reduce the susceptibility of the grain aphid *Sitobion avenae* to two different species of parasitoids, although parasitoid females did appear to preferentially oviposit into uninfected hosts. More recently, and most relevant to my study, Sochard et al. (2020) assessed the effects of different strains of *H. defensa* on both parasitism resistance and defensive behaviours against parasitoids in pea aphids. They found that different strains of *H. defensa* provided various levels of protection against parasitism and varied in their effects on aphid behaviours. While some strains reduced all considered behavioural defences, consistent with earlier studies (Dion et al., 2011, Polin et al., 2014), other strains reduced aggressiveness only if they protected their hosts completely against parasitoids. Across the associations and lineages examined, reduced frequencies of aphid defensive behaviours were not found to be related to the level of resistance conferred by the secondary symbionts (Sochard et al., 2020), although future works investigating more symbiont-lineage associations will offer broader insight into correlations between symbiont-mediated phenotypes. Sochard et al. (2020) suggested that, given that symbiont effects on defensive behaviours and resistance to parasitism were not linked, the reduction of behaviours in infected aphids could simply be a by-product of the infection. Again, it would be interesting to discover whether the antipredator defensive behaviours of aphids infected with different strains of *H. defensa* (offering varying levels of parasitism-resistance) vary depending on predator type. Given that the mechanism of protection offered by parasitism resistance is unlikely to be a mechanism that also offers protection from predators, this would help unpack whether symbiont effects on behaviour relate to the level of parasitism resistance they provide or whether changed behaviours are by-products of infection. The reduction of defensive behaviours should be counter-selected if it increases susceptibility to predators (Polin et al., 2014) or parasitoid genotypes or species unaffected by *H. defensa* in their hosts (Asplen et al., 2014, McLean and Godfray, 2015); but Sochard et al. (2020) highlight the need for field studies testing the consequences of reduced defensive behaviours due to *H. defensa* for aphid survival and fitness following natural predator-prey encounters.

In light of the work by Sochard et al. (2020), it is possible that my results regarding infected pea aphids' use of different behaviours and relative susceptibility to predation differ from earlier literature (Dion et al., 2011, Polin et al., 2014) because of some variation in the pea aphid biotypes tested or strains of *H. defensa* present. Importantly, I did not assess the level (or specificity) of parasitism-resistance that the strain of *H. defensa* provided my infected pea aphids with, or whether this related to the pea aphid biotype, meaning that I could not confidently suggest that the *H. defensa* here would definitely act as a ‘protective symbiont’ against parasitoids. If the symbiont strain in this study offered little or no protection from parasitism, then infected aphids would not be expected to exhibit reduced defences (theoretically adaptive if parasitism-resistant individuals can reduce the costs and energetic expenditure of defences) unless such a reduction was merely a by-product of infection. Whether the symbiont strain offered some protection against parasitism or not, infected pea aphids in this current study showed a reduced use of evasive responses, but this did not appear to increase susceptibility to predation – in contrast to previous work (Polin et al., 2014). This is unlikely to be because the mechanism of protection against parasitism is the same as the mechanism of protection against predation (if *H. defensa* did indeed act as a ‘protective symbiont’ against parasitoids). It might, instead, be that the strain of *H. defensa* in the infected pea aphid line used in this study reduced dropping behaviour but not aggressiveness, relative to the cured strain, enabling aphids to still defend themselves sufficiently. However, my study had a relatively small sample size and a short timescale, so it is possible that bigger-scale or longer-term effects of *H. defensa* on aphids’ abilities to evade predation were not captured. Sochard et al. (2020) argue that conferring some protection against parasitism should not be enough for a symbiont to be labelled ‘protective’, rather it should “[limit] the development of the enemy without reducing its host’s fitness drastically”. The full effects of symbionts on host fitness and behaviours during encounters with all their common natural enemies must be considered before its overall benefits and costs to its host can be determined. *H. defensa* infection has also been found to have detrimental effects on host fitness in aphids by reducing host survival and reproductive success (Oliver et al., 2006, Simon et al., 2011, Vorburger and Gouskov, 2011, Cayetano et al., 2015).

One component that also could be valuable in understanding the role of *H. defensa* infection in its hosts is identifying whether any effects it appears to have on behaviour in other aphid species also are due to its presence alone or more generally due to the parasitism resistance it confers. To contribute to this knowledge, my study also tested potato aphids, a species for which there has been little evidence for *H. defensa* providing protection from parasitism; instead, parasitism resistance is associated with aphid genotype (Clarke et al., 2017). The genetic backgrounds and infection statuses of all four potato aphid lines used in my study originated from individuals sampled in nature, so no artificial elimination or transfection manipulations were necessary to compare differences between the behaviours of aphids infected with *H. defensa* or uninfected, within or between genotypes that were either parasitism-resistant or -susceptible. However, following subsequent checks on the potato aphid lines, I have reason to think that an unknown fraction of the RB15/11 aphids used in this experiment may not have been infected with *H. defensa*, and so the following results regarding infection status are conservative, since I implicitly assumed that all individuals of this line used in my experiment were infected. As with the pea aphids, all four lines were reared under identical conditions and tested with the two predator types: ladybird adults and lacewing larvae.

*H. defensa* infection status, predator type and aphid genotype (parasitism-resistant or -susceptible) all appeared to influence potato aphid defences, with a possible interaction between infection status and predator type having a significant influence on some behavioural components of potato aphids' antipredator repertoire. Across both parasitism-resistant and parasitism-susceptible genotypes, symbiont-infected potato aphids showed greater differentiation in the frequency of behaviours (particularly evasive behaviours) exhibited in response to the two predator types than the uninfected lines did. As *H. defensa* does not provide strong protection to potato aphids from parasitism (Clarke et al., 2017), these findings indicate that the presence of the symbiont itself, rather than any influence it may have on parasitism resistance, may be a key determinant of defensive behaviours in aphids. This supports the suggestion of Sochard et al. (2020) that changes in aphid behaviour as a result of infection by *H. defensa* are by-products of the infection, they are not linked with the parasitism resistance sometimes associated with the symbiont in pea aphids. As mentioned above, though, I cannot be confident that all of the RB15/11 line potato aphids used in this experiment were infected with *H. defensa*, so these findings require strengthening with further testing. It is also worth noting that, regardless of genotype or symbiont infection, predator type had the most significant effect when the proportion rather than frequency of evasive behaviours were considered; as with the pea aphids, ladybird adults elicited a higher proportion of dropping and walking away than did lacewing larvae. This again supports the idea that different predator types have different foraging styles, which are responded to differently by aphids (Brodsky and Barlow, 1986); this idea is explored further in Chapter Five.

Whether behavioural effects of *H. defensa* are linked to parasitism resistance or not, the specific mechanisms by which symbionts could affect aphid host behaviours remain unknown. In other species, such as the fruit fly *Drosophila melanogaster* various commensalisitic gut microbes have been suggested to influence behavioural patterns, including mating preferences, locomotor activities, and food choice (Hosokawa and Fukatsu, 2020). Microbiota appear to affect mating preference of flies by altering the levels of sex pheromones in the cuticular hydrocarbon of hosts (Sharon et al., 2010), but the specific signals and compounds involved have not been identified. Concerning locomotor activity, it is likely that microbe-associated influences on metabolism modulate host physical activity, but the exact neurons, neuronal mechanisms, and potential changes in firing patterns involved also remain to be identified (Schretter et al., 2018). Similarly, while nutrient-sensing pathways and olfactory receptors have been implicated in microbiota influences on host food choice (Leitão-Gonçalves et al., 2017, Wong et al., 2017), the specific products and molecular biology at play are yet to be explored in-depth. These suggestions concerning the influence of gut microbiota on *Drosophila* behaviour resemble what is known about the influence of facultative symbionts in aphids on their host behaviour, in that we only have theories as to their mechanism thus far. For some aphid symbionts, there are more suggestions regarding their influence on host physiological abilities than behaviour. For example, symbiont-mediated protection from heat stress has been hypothesised to be linked to upregulation of stress-response genes, immune cells, and heat-shock proteins (see Heyworth et al. [2020] for references).

Regarding physiological influences of *H. defensa* specifically, several studies have attempted to elucidate the mechanisms behind the protection from parasitism certain strains of it provide to some aphid species against particular parasitoids. Parasitism protection is thought to arise from infection of *H. defensa* with bacteriophages called APSEs (an acronym stemming from '*A. pisum* secondary endosymbiont'). These phages are believed to carry several toxin-encoding genes that provide immunity to host aphids by preventing the development of parasitoid larvae (Moran et al., 2005, Brandt et al., 2017, Oliver and Higashi, 2019, Purkiss et al., 2021). However, understanding of the role of symbionts in aphid-parasitoid interactions has only recently been made possible by advances in molecular and genomic methodologies, and further research is needed in order to identify the particular factors that damage wasp development, and to explain how specific delivery of toxins to wasp larvae is achieved without causing harm to the aphid host (Su et al., 2013, Oliver and Higashi, 2019). Even if such details are elucidated in future investigations, it may not be obvious what factors or pathways are associated with symbiont-mediated behavioural variation. While reduced behaviours were posited by (Dion et al., 2011) and (Polin et al., 2014) as being potentially adaptive to pea aphids with *H. defensa*-associated parasitism protection (through its minimising of costly and potentially unnecessary defences), it is unclear how the protection offered by phage-borne toxins would either be sensed by the aphid host or directly influence pathways associated with behaviour. Moreover, if behavioural variation associated with infection with *H. defensa* is not in fact related to the level of parasitism protection it offers (Sochard et al., 2020), changes in behaviour must relate to alternate neurobiological and/or molecular mechanisms.

A recent study by Badji et al. (2021) investigated whether infection with *H. defensa* alters antennal sensitivity of pea aphids to alarm pheromone. If *H. defensa* presence was associated with higher antennal detection threshold of alarm pheromone and a decrease in evasive responses towards natural enemies, this could help to explain the previously reported reduction in evasiveness and aggression in infected aphids (Dion et al., 2011, Polin et al., 2014). However, Badji et al. (2021) found no significant influence of symbiotic status on antennal sensitivity to alarm pheromone, indicating that *H. defensa* does not influence pea aphids' olfaction of aversive cues. Further studies, such as that of Badji et al. (2021), purpose-designed to examine the effects of symbiont infection on behaviour, and what may drive such effects, are necessary. Possibly, as has been suggested for some microbiota in *Drosophila* (Schretter et al., 2018), *H. defensa* presence in aphids affects the firing (or reduction in firing) of particular sensory or locomotor neurons, resulting in altered behaviours. However, science has only recently begun to appreciate the role of symbionts in multi-trophic interactions and it is likely through the development of more advanced techniques over the coming decade that light will be shed on the mechanistic basis of such interactions. Whether the mechanism by which a symbiont influences host behaviour influences its susceptibility to predation, palatability to predators, and the effect its consumption has on predators should also be explored further for a great range of predator-prey systems, as this will carry important consequences for the population dynamics and evolution of both sides.

Despite potato aphids' defensive behaviours, 17 individuals were consumed during trials. However, neither symbiont infection status nor genotype (parasitism resistance or susceptibility) appeared to consistently affect vulnerability to predation, as captured aphids included individuals from every infection status-genotype combination. Again, this differs from the finding of Polin et al. (2014) that pea aphids harbouring *H. defensa* suffered higher predation as a consequence of them exhibiting reduced defences, but the potato aphids here did not exhibit such a clear behavioural trend associated with the symbiont. Additionally, the fact that no infection status-genotype combination experienced a significantly greater risk of predation suggests that although some components of any aphid line's defensive repertoire may be reduced relative to others, this alone does not indicate that their overall defensive response is reduced – they may increase their use of alternative behaviours, or use more costly defences sparingly but effectively. In order to build a full picture of aphid behavioural ecology, the full suite of available behaviours utilised during encounters with predators needs to be considered. The longer-term consequences of predator-prey interactions involving prey-hosted symbionts should also be examined in light of work by Costopoulos et al. (2014), that suggests the facultative symbionts *S. symbiotica* and *H. defensa* may indirectly defend their hosts' clonal descendants against predation. While these symbionts did not deter the feeding of *Hippodamia convergens* ladybird larvae on infected pea aphids, they were found to decrease the subsequent survival from egg hatching to pupation and affect weight at adult emergence (Costopoulos et al., 2014). Whether symbiont infection increases susceptibility to predation (Polin et al., 2014) or not, as in the study I describe here, the lifetime fitness of aphids might benefit from infection if their offspring experience greater reproductive success in an environment with fewer surviving predators; future studies examining the influence of consuming symbiont-infected aphids on adult ladybird fecundity may add to this.

Dropping in particular, and the associated costs it carries for individuals, often remains unappreciated as an antipredator defence; what is known about dropping is reviewed in detail in Chapter Three. Pea aphids are far more well-known for using dropping behaviour than potato aphids, but the species differences in dropping propensity could not be fairly compared here, as their assays were not conducted alongside one another and the aphids of each species were likely at slightly different developmental stages. Nonetheless, while pea aphids certainly exhibited more dropping than potato aphids in this study, dropping was a substantial component of the potato aphids' defensive repertoire. There is a need for comparative studies of aphid antipredator behaviours to explore other aphid species' dropping behaviour in order to fully understand its costs and benefits under different conditions and its evolution – potato aphids are perhaps a useful candidate for this. Additionally, studies investigating the behaviours of aphids at different stages throughout their life cycle will be valuable. Although immature aphids, being more vulnerable, may be more conservative in their defensive behaviours (Roitberg and Myers, 1978, Gish et al., 2012), my study intentionally used immature individuals, in order to prevent the populations from growing throughout the experiment. Whether symbiont-mediated phenotypes or responses to different predator types vary significantly depending on aphids' developmental stage is worthy of further investigation.

Given the significant economic impact aphids can have on agricultural production (Dedryver et al., 2010), increasing understanding of the factors that affect their behaviours and subsequent fitness is important as a guide for effective management strategies. In particular, defensive symbioses are of interest where they have the potential to compromise the effectiveness of biological control with parasitoids (Oliver et al., 2005, Vorburger, 2018), and here understanding how such symbioses affect interactions with other aphid natural enemies (i.e. predators) could be invaluable when devising effective biocontrol strategies. By exploring the influence of *H. defensa* infection on the antipredator defensive behaviours of two major pest species of aphid, this work aimed to contribute to current understanding with regards to the association of pea aphids with *H. defensa* and offer novel findings about the behaviours seen in potato aphid lines with and without the symbiont. Regardless of symbiont infection, both aphid species tested here differentiated their defensive behaviours depending on the type of predator they encountered. The frequency and types of responses a predator elicits can have a significant, indirect influence on a prey individual's survival chances and subsequent fitness, leading to knock-on consequences for pest populations; this is particularly true of costly defences such as dropping. Facing aphids with predators enables observation of aphid antipredator behaviours that can be considered important non-consumptive effects of natural enemies due to their subsequent influence on aphid fitness (Nelson and Rosenheim, 2006). The methodology utilised in this chapter, wherein aphids experienced encounters with foraging predators on live plants, also formed the basis of the next two chapters. Chapter Five and Six, respectively, further explore the influence of predator type on the specific antipredator behaviours used by aphids and the behaviours exhibited by aphids after dropping to escape foliar-foraging predators; such behaviours could significantly influence fitness and be considered as indirect effects of those predators. Future studies that also adopt methodologies involving live encounters between predators and prey under naturalistic conditions will continue to be of great use in developing biological pest control and informing selection of the most effective natural enemies.

# Chapter 5: Flexible use of dropping behaviour by aphids

Material from this chapter formed the basis of the publication: Humphreys, R. K., Ruxton, G. D. & Karley, A. J. 2021. Drop when the stakes are high: adaptive, flexible use of dropping behaviour by aphids. Behaviour, 158(7), pp.603-623.

For herbivorous insects, dropping from the host plant is a commonly-observed antipredator defence. This chapter reports further findings from the experiment described in Chapter 4. The use of dropping compared to other behaviours and its timing in relation to contact with a predator was explored in both pea aphids (*Acyrthosiphon pisum*) and potato aphids (*Macrosiphum euphorbiae*). Pea aphids dropped more frequently in response to ladybird (*Adalia bipunctata*) adults than lacewing (*Chrysoperla carnea*) larvae. Potato aphids mainly walked away or backed-up in response to both predator types; but they dropped more frequently relative to other non-walking defences when faced with ladybird adults. Contact with a predator was an important influencer of dropping for both species, and most drops occurred from adjacent to the predator. Dropping appears to be a defence adaptively deployed only when the risk of imminent predation is high; factors that increase dropping likelihood include presence of faster-foraging predators such as adult ladybirds, predator proximity, and contact between aphid and predator.

## 5.1 Introduction

The immediate risk of predation any prey individual faces will vary greatly over the course of its lifetime, changing on seasonal, daily and even shorter timescales (Lima and Dill, 1990). As I established in Chapter One, and developed further in Chapter Three, the effectiveness of any behavioural antipredator defence at enhancing a prey individual’s fitness can be expected to vary depending on the context of specific predator-prey interactions, and so these adaptations should be flexibly deployed. Behavioural decision-making should reflect the trade-offs between the relative costs and benefits of any behaviour compared to the alternatives available to a prey individual, the evaluation of which will depend on a wide range of ecological variables.

Dropping is an antipredator behaviour that is common and taxonomically widespread across the animal kingdom. As I described in Chapter Three, it involves the passive or active release from a substrate (or loss of powered motion) such that an animal's escape from an imminent threat is powered by gravity, wind or water currents. The obvious benefit of such a defence is immediate and rapid escape from the perceived threat (Losey and Denno, 1998a), but this behaviour can also carry short- and long-term fitness costs to the dropped individual such as: exposure to new predators (Losey and Denno, 1998a), exposure to harsher environmental conditions (Ruth et al., 1975, Roitberg and Myers, 1979), reduced feeding time (Roitberg et al., 1979, Johnson et al., 2007), increased development time (Agabiti et al., 2016), and reduced lifetime fecundity (Nelson et al., 2004, Nelson and Rosenheim, 2006, Nelson, 2007, Agabiti et al., 2016). A display of tonic immobility immediately after dropping might serve to reduce localisation by predators (Miyatake et al., 2009) but could also increase costs by lengthening exposure to harsher conditions and time without feeding; this idea is explored further in Chapter Six. Dropping has been studied most commonly in aphids (see Chapter Two and the Appendix for details on aphid biology), a group for which several abiotic and biotic factors have already been demonstrated to influence the trade-offs associated with the decision to drop; see Chapter Three and key references therein (Dill et al., 1990, Losey and Denno, 1998a, 1998b, Braendle and Weisser, 2001) for examples.

Characteristics of predators that influence the propensity to drop in various aphid species are: the relative size of a predator compared to its prey (Brown, 1974, Evans, 1976a, Losey and Denno, 1998a, Francke et al., 2008, Hoki et al., 2014), the predator species identity (Brown, 1974, Brodsky and Barlow, 1986, Losey and Denno, 1998a, Day et al., 2006, Hoki et al., 2014), and the predator's movement and/or foraging style (Brown, 1974, Brodsky and Barlow, 1986, Losey and Denno, 1998a, Day et al., 2006, Francke et al., 2008). For example, Francke et al. (2008) showed that older ladybirds (*Harmonia axyridis*) , which were also larger and more active than younger ladybirds, induced more frequent dropping by pea aphids. Losey and Denno (1998a) compared predator types and found that the coccinellid *Coccinella septempunctata* foraged more energetically than two smaller heteropteran predators, and initiated more than three-fold greater dropping by pea aphids. The underlying mechanisms for the links between predator size and activity and prey dropping behaviour may be the intensity of substrate vibration produced by the foraging predator, the encounter rate with aphids, the volume of aphid alarm pheromone released, or a combination of these (Montgomery and Nault, 1977, Clegg and Barlow, 1982, Francke et al., 2008). Additionally, predators sometimes become smeared with alarm pheromone (Mondor and Roitberg, 2004), and movement of these predators will also be expected to increase aphid dropping (Francke et al., 2008).

Variability in the occurrence of dropping behaviour alone is not enough to fully evaluate a prey individual’s antipredator response; the use of dropping might depend on an aphids’ ability to use alternative defences. Aphids possess morphological and physiological defence adaptations, such as tube-like siphunculi on the abdomen which secrete waxy droplets and alarm pheromone. Droplets harden upon contact and can bind the appendages and mouthparts of predators and parasitoids (Dixon, 1958, Edwards, 1966, Outreman et al., 2005, Butler and O'Neil, 2006, Pickett and Glinwood, 2007, Vandermoten et al., 2012). Some aphids also possess defensive structures such as frontal horns that can be used to attack predators (Arakaki, 1989, Pickett and Glinwood, 2007). Indirect defence also occurs for some aphids – myrmecophilous species form mutualisms with ants, which deter predators, and consequently exhibit reduced predator-avoidance behaviours (Depa et al., 2020). The deployment of behavioural defences can be more flexible, however, in response to the context of predation than morphological adaptations and indirect defences, and so the trade-offs associated with dropping will most valuably be weighed-up relative to alternative defensive behaviours. As well as dropping (Dixon, 1958, Roitberg and Myers, 1978, Clegg and Barlow, 1982, Agabiti et al., 2016, Harrison and Preisser, 2016), aphids’ behavioural responses to perceived predatory threat include: remaining motionless to avoid detection (Dixon, 1958, Dixon, 1985, Brodsky and Barlow, 1986), kicking (Dixon, 1958, Hartbauer, 2010, Dion et al., 2011, Polin et al., 2014), attacking with frontal horns (Arakaki, 1989), and walking away or backing-up (Dixon, 1958, Clegg and Barlow, 1982, Brodsky and Barlow, 1986). Some studies have already considered this broader portfolio of behaviours and found that when faced with larger, more mobile predators, dropping may be the most effective means of escape; but against the attacks of smaller, less-active predators, aphids might be able to survive by exhibiting less costly behaviours such as kicking or walking away (Brown, 1974, Evans, 1976a, Roitberg and Myers, 1978). For example, Brodsky and Barlow (1986) examined the defensive responses of pea aphids to syrphid larvae and coccinellid beetles. Syrphid larvae foraged more slowly than coccinellid beetles and, in response, aphids primarily backed-up. By contrast, coccinellid beetles foraged more vigorously, causing greater vibrations on the host plant, and most aphids that they encountered dropped off the plant, especially at higher temperatures when beetles were most active (Brodsky and Barlow, 1986). Variable responses to different predator types was also an important finding of the analysis presented in Chapter Four, but I examine this idea more closely in this current chapter. Rather than individuals exhibiting a blanket defensive response against any threat, aphids can respond flexibly depending on the foraging rate, stage of development, method of attack, or species of a predator, and on the abiotic conditions.

Additional aspects of predator-prey interactions that could influence a prey individual's threat evaluation, and therefore propensity to utilise more extreme antipredator defences, include the proximity of the predator and whether the predator has made contact with the individual. Although many studies have explored the responses of aphids to attack, sometimes only behaviours that occur following physical contact with a natural enemy are recorded (Evans, 1976a, Dion et al., 2011, Polin et al., 2014). Although contact can certainly be an important trigger for defensive behaviours, including dropping (Roitberg and Myers, 1979, Brodsky and Barlow, 1986, Dill et al., 1990, Losey and Denno, 1998a, Dion et al., 2011), defence behaviours - including dropping - can be initiated ahead of any contact (Clegg and Barlow, 1982, Hajek and Dahlsten, 1987, Minoretti and Weisser, 2000, Nelson and Rosenheim, 2006, Harrison and Preisser, 2016, Gish, 2021). As touched on earlier, and in Chapter Three, plant-borne vibrations and alarm pheromone detection can serve as alternative, or additional, triggers for dropping in aphids (Clegg and Barlow, 1982, Losey and Denno, 1998a, Schwartzberg et al., 2008, Hartbauer, 2010, Gish, 2021), and dropping in many taxa is known to take place at different stages of the predation sequence, as either a primary (pre-contact) or secondary (after physical contact with the predator) defence. Yet studies do not tend to differentiate between pre- and post-contact dropping by aphids, although this could be important to understanding the threat level at which the decision to drop is taken. Similarly, where aphid dropping occurs in relation to a foraging predator is not often recorded, even though this would be expected to affect the sense of imminent threat experienced by the prey.

This current study utilised additional data collected during the experiment described in Chapter Four (that also forms the basis of the study in Chapter Six), exploring a range of defensive behaviours exhibited by pea aphids and potato aphids in response to two predator types with different foraging styles: two-spot ladybird (*A. bipunctata*) adults and lacewing (*C. carnea*) larvae. Many studies record only the use of dropping as a defensive behaviour by aphids, but here I consider its use relative to alternative behaviours that may still be effective but less costly. Further, the defensive behaviour portfolios of aphids in response to different predatory threats have not been extensively explored for aphid species other than pea aphids, so here I also investigate potato aphids. Both the pea and potato aphid are in the Tribe Macrosiphini (Stekolshchikov and Buga, 2020) but – as I describe in Chapter Two and the Appendix – while the pea aphid specialises on legumes, the potato aphid is highly polyphagous and feeds on plants in many families (van Emden and Harrington, 2007, AHDB, 2015). The two species also differ in the timing and stages of their life histories (AHDB, 2015), but both are commercially-important crop pests and virus vectors (van Emden and Harrington, 2007). The differences in the biology and life history of the potato aphid make it a good model species to compare with its close relative: the pea aphid.

As part of exploring aphids' assessment of risk based on factors such as predator type, proximity and contact, and the use of dropping relative to other behavioural options, this chapter aimed to test two hypotheses:

1. Different predator types vary in their movements, mobility, and attack style. Therefore, I predict that the searching behaviour of ladybird adults will cause more frequent dropping by aphids than that of lacewing larvae, and that aphids will show differential use of various behavioural responses to the two predator types.
2. Dropping is the costliest defensive strategy for aphids, and as such it should only be used when threat is high-risk and imminent. Therefore, I predict that contact is an important trigger for dropping and that dropping will occur most commonly when a predator is in close proximity.

## 5.2 Materials and methods

The data collection for this chapter took place at the James Hutton Institute (Dundee, UK), as part of the experiment detailed in Chapter Four. Briefly, the antipredator behaviours of 6-7 d old 3rd-4th instar pea aphids and 8-9 d old 3rd-4th instar potato aphids in response to ladybird adults and lacewing larvae were recorded on live, immature, potted plants. A total of 16 pea aphid assays took place in May 2019, eight with lacewing larvae as predators and eight with ladybird adults as predators, using pots of 2-4 week old bean cv. The Sutton plants. A total of 32 potato aphid assays took place in July 2019, 16 with lacewing larvae as predators and 16 with ladybird adults as predators, using pots of 2-3 week-old potato cv. Desirée plants. The plant material, insect rearing and maintenance, and experimental protocol were as those described in detail in Chapter Four, with the exception of the following additional observations made for the purpose of the study in this chapter. When antipredator drops by aphids occurred, it was noted whether that aphid had been contacted by the foraging predator and from what proximity to the predator the aphid dropped (immediately beside, same immediate plant section, same gross plant section, not close by but from same plant, or from the other plant entirely); see Figure 5.1 for a diagrammatic representation of these proximities.

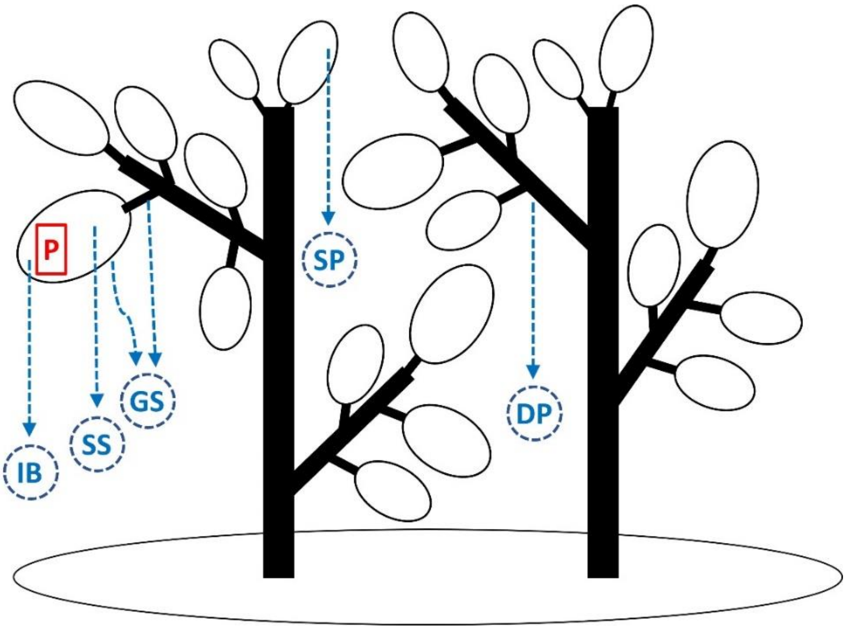

**Figure 5. 1:** Diagram showing the different proximities aphids were classified as dropping from with respect to the predator present (represented by the red, boxed ‘P’). Dropping is indicated by dashed arrows, and the dashed circles labels represent the following proximities: IB = immediately beside the predator on the same plant section, SS = same immediate plant section as the predator (i.e. same leaf upperside, leaf underside, section of stem, petiole, or petiolule), GS = same gross plant section as the predator (which included both sides of any individual leaf and its immediately connecting petiolule), SP = same plant but not on the same gross plant section as the predator, and DP = different plant from the one where the predator was present.

### 5.2.1 Analysis

All statistical analyses were conducted using R version 4.0.3 (R Core Team, 2020). The analysis for Hypothesis 1 first involved a 2 x 5 extension of Fisher’s exact test using the total counts of each response type exhibited with the two different predators. Standardised residuals were then calculated (Sharpe, 2015) for the responses to each predator type, using expected counts derived from the relative proportions of responses that the other predator type provoked. The predator types were then considered separately, using one-way ANOVAs (one-way analysis of means, not assuming equal variance) and post-hoc Games-Howell tests (suitable for unequal group sizes and variances) to compare the mean proportions of different responses per trial. The analysis for Hypothesis 2 involved three chi-square goodness-of-fit tests, comparing the frequencies of: 1) pre- and post-contact drops, 2) post-contact drops from different proximities to predators, and 3) pre-contact drops from different proximities to predators.

## 5.3 Results

### 5.3.1 Hypothesis 1: Ladybird adults cause more frequent dropping than lacewing larvae, and aphids show differential use of behavioural responses to the two predator types.

First, I considered the propensity of pea aphids to use different defences in response to the two predator types. A significant difference was found between the raw counts of aphid responses to ladybirds (drops: 61, walks: 69, kicks: 71, shifts: 18, and no response: 0) and to lacewings (drops: 4, walks: 41, kicks: 73, shifts: 15, and no response: 0) (P <0.001). Comparison of the relative magnitudes of the standardised residuals indicated that lacewing larvae elicited significantly fewer drops (std. res. = -5.43) but significantly more kicks (std. res. = 4.55) than would be predicted under the null hypothesis that pea aphids reacted to lacewing larvae in the same way as they reacted to ladybird adults. Correspondingly, ladybird adults were found to elicit significantly more drops (std. res = 21.20) but significantly fewer kicks (std. res. = -4.49) than would be predicted under the null hypothesis that pea aphids reacted to them in the same way as they reacted to lacewing larvae.

Focusing first on pea aphid responses to lacewing larvae, a significant difference was found in the mean proportion per trial of drops (9%), walks (40%), kicks (40%), and shifts (11%) exhibited by aphids (Figure 5.2a, F = 5.40, num df = 3.0, denom df = 13.9, P <0.05); there were no cases of no response to contact. Between the proportions of these defences, aphids showed significantly more kicks compared with shifting (P <0.05). Turning to the responses to ladybird adults, a significant difference was also found in the mean proportion per trial of drops (29%), walks (35%), kicks (26%), and shifts (10%) exhibited by aphids (Figure 5.2a, F = 7.03, num df = 3.0, denom df = 15.0, P <0.01); again there were no cases where pea aphids did not respond to contact with a predator. Between these defences, aphids showed significantly more dropping (P <0.05) and walking (P <0.01) than they did shifting behaviours.

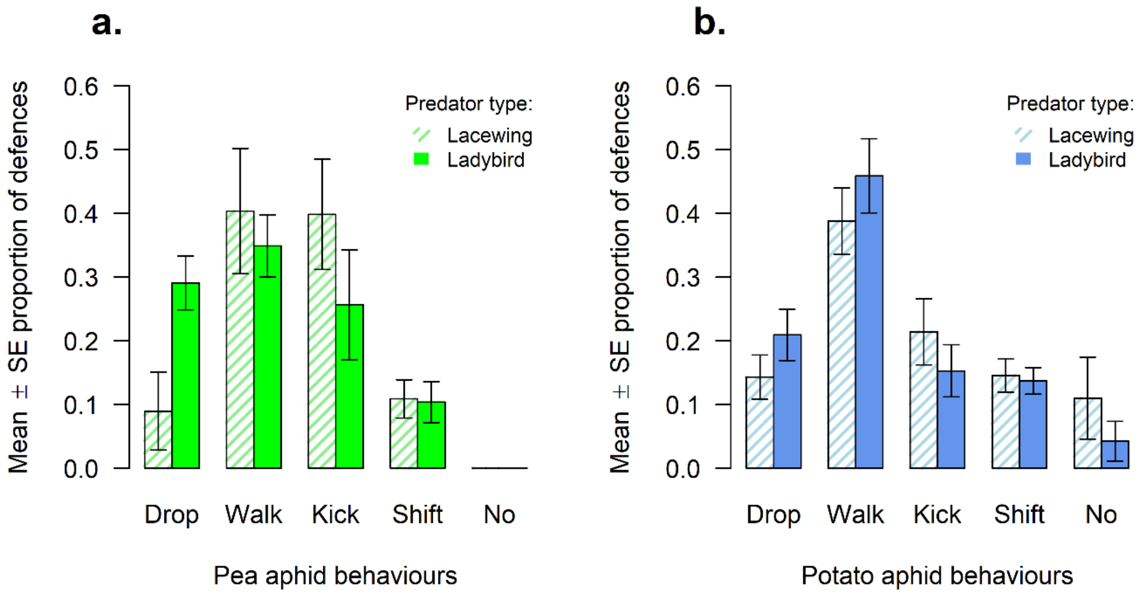

**Figure 5. 2:** The mean ± SE proportion of total defensive behaviours categorised as dropping, walking away/backing-up, kicking, shifting, and remaining motionless after contact exhibited by aphids in trials, where **a)** shows the pea aphid trials with lacewing larvae (n = 8, dashed pale green bars) and with ladybird adults (n = 8, solid darker green bars), and **b)** shows the potato aphid trials with lacewing larvae (n = 16, dashed pale blue bars) and with ladybird adults (n = 16, solid darker blue bars).

Next, the propensity of potato aphids to use different defences in response to the two predator types was explored. As with the pea aphids, the proportion of raw counts of each defence was considered first. A significant difference (P <0.05) was found between aphids' relative use of different responses to ladybirds (drops: 68, walks: 154, kicks: 81, shifts: 52, and no response: 4) and to lacewings (drops: 27, walks: 72, kicks: 65, shifts: 27, and no response: 5) . In response to lacewing larvae, potato aphids were found to kick more than would be predicted (std. res. = 3.12) based on expected values generated from the counts of behaviours in response to ladybird adults. In response to ladybird adults, potato aphids were found to drop more (std. res. = 2.64) and kick less (std. res. = -3.49) than would be predicted based on expected values generated from the counts of behaviours in response to lacewing larvae.

Considering responses to lacewing larvae only, a significant difference was found in the mean proportion per trial of drops (14%), walks (39%), kicks (21%), shifts (15%) and lack of response to contact (11%) exhibited by potato aphids (Figure 5.2b, F = 4.77, num df = 4.0, denom df = 36.5, P <0.01). Between these defences, potato aphids showed significantly more walking than dropping (P <0.01), shifting (P <0.01), and no response to contact (P <0.05).

Looking next at responses to ladybird adult predators, a significant difference was found in the mean proportion per trial of drops (21%), walks (46%), kicks (15%), shifts (14%) and lack of response to contact (4%) exhibited by potato aphids (Figure 5.2b, F = 10.10, num df = 4.0, denom df = 36.3, P <0.001). Between these defences, potato aphids showed significantly more walking than they did dropping (P <0.05), kicking (P <0.01), shifting (P <0.001), and no response to contact (P <0.001). Interestingly, though, in response to contact with foraging ladybirds, potato aphids also showed significantly more dropping than no response (P <0.05). In response to both predators, then, walking away was the most prevalent defence of potato aphids in terms of raw occurrence. But in response to ladybirds, aphids dropped significantly more than they showed no response to contact (the cases of no response to contact translated into those aphids being grabbed by predators).

### 5.3.2 Hypothesis 2: Contact is an important trigger for dropping, and dropping will occur most commonly when a predator is in close proximity.

Considering the dropping behaviour deemed to result from nearby predator presence, pea aphids dropped significantly more commonly post-contact (n = 44) than pre-contact (n = 5) ($\chi^2_1 = 31.0$, P <0.001). Of the cases where pea aphids dropped post-contact with a predator, 39 occurred from adjacent to the predator, three from the same plant section as the predator, and two from the same gross plant section as the predator. A significant difference was found between the frequencies of post-contact drops from these three recorded proximities ($\chi^2_2 = 60.6$, P <0.001), with the vast majority of drops starting from immediately beside the predator. There were only five cases of pea aphids dropping pre-contact with a predator: four of these aphids dropped from the same plant section as the predator and one dropped from the same gross plant section. The small sample size meant that a significant difference could not be detected between the frequency of pre-contact drops from these two proximities ($\chi^2_1 = 1.8$, P = 0.18), but it seemed that when aphids dropped pre-contact it was primarily from the same plant section as the predator.

Turning to the potato aphids, considering the dropping behaviour deemed to result from nearby predator presence, potato aphids also dropped significantly more commonly post-contact (n = 77) than pre-contact (n = 11) ($\chi^2_1 = 49.5$, P <0.001). Of the cases where potato aphids dropped post-contact with a foraging predator: seventy drops occurred from immediately beside the predator, five from the same plant section as the predator, and two from the same gross plant section as the predator. A significant difference was found between the frequencies of post-contact drops from these three recorded proximities ($\chi^2_2 = 115.0$, $P < 0.001$), with the vast majority of drops starting from immediately beside the predator. There were only eleven cases of potato aphids dropping pre-contact with a predator, but where the dropping was still interpreted as being in response to the presence of the predator as opposed to some other unknown trigger: eight of these aphids dropped from the same plant section as the predator, two dropped from the same gross plant section, and one dropped from right beside the predator. A significant difference was found between the frequency of pre-contact drops from these three proximities ($\chi^2_2 = 7.8$, $P < 0.05$). Even though the small sample sizes meant that this test might be unreliable, it illustrates a tendency shown by potato aphids that pre-contact drops occurred primarily from the same plant section as the predator, as was shown for pea aphids.

## 5.4 Discussion

Overall, the study collected data on the range of behaviours both pea and potato aphids exhibit in response to two major predatory threats, finding that dropping was an important component within both aphids’ defensive behavioural repertoires. Considering first Hypothesis 1, both species dropped significantly more commonly (relative to other defences) when faced with ladybird adult predators than lacewing larvae predators – lacewing larvae correspondingly elicited a significantly greater proportion of kicking defence. This supports the previous finding from Chapter Four that predator type is a major influence on the antipredator defences used by aphids. In pea aphids, dropping made up a similar proportion of the total behavioural responses as walking away and kicking in response to ladybird adults, but dropping occurred significantly less frequently when aphids were faced with lacewing larvae: drops constituted around a three times smaller proportion of the total defensive behaviours exhibited and occurred at a fifteen times smaller raw frequency. Pea aphids rarely shifted when faced with either predator, and never remained motionless after any predator contact. In potato aphids, dropping made up a similar proportion of the total behavioural responses as kicking, but a significantly smaller proportion than walking away in response to ladybird adults. Dropping made up a similar proportion of responses as shifting and remaining motionless after contact in response to lacewing larvae, where again walking away was by far the most common response. Although walking away was the primary response of potato aphids to both predator types, dropping still constituted an important component of their defensive response and differentiation in its usage was still seen between the two predator types.

Predator species (Brown, 1974, Harmon et al., 1998, Hoki et al., 2014) and predator functional types (Brodsky and Barlow, 1986, Losey and Denno, 1998a, Day et al., 2006) vary in the way that they search plants and thereby elicit different responses in their prey. Pea aphids have previously been found to drop more frequently from host plants in response to coccinellid beetles compared to syrphid larvae, an effect that was attributed to the energetic and vibrational differences in predator foraging styles (Brodsky and Barlow, 1986). In my study, lacewing larvae predators appeared to match more closely the syrphid larvae style of foraging, in that they were generally slow-moving, while ladybird adults fitted the typical behaviour of coccinellids being fast-paced predators. This might explain why dropping made up a greater proportion of both aphid species' responses to ladybird adults – these predators were more energetic, perhaps causing more vibrations across plants, and more likely to attack quickly, therefore the most extreme defence of dropping might be more necessary to evade capture. By contrast, with slower-foraging lacewing larvae there might have been fewer plant vibrations, attacks were perhaps less sudden, and other defences such as walking away or kicking might be effective without aphids resorting to dropping.

Viewed in conjunction with the finding from Chapter Four that ladybird adults elicited a higher proportion of evasive responses (that is, dropping and walking away) than did lacewing larvae, predator type (or at least foraging style) does seem to be an important factor that influences dropping propensity, and likely feeds into aphids' assessment of predation risk. Additional experiments would be needed to confirm that the differing reactions of aphids to these two predator types were a consequence of these predator characteristics rather than morphological or chemical signalling traits. Recent work comparing the avoidance and dropping behaviour of pea aphids in response to native and invasive ladybird species indicates that large body size and high activity levels are indeed the key predator characteristics that trigger dropping. Even though predator naivety means that the chemical cues of non-native ladybirds do not provoke avoidance behaviour (Bertleff et al., 2020, Ünlü et al., 2020a), dropping rates in response to the non-native species were found to be comparable to those in response to the native species; presumably because the most important cues for prey risk assessment were present in both foraging predators (Ünlü et al., 2020b). However, these studies compared responses to coccinellids alone. Future studies that examine responses to more varying predator types with comparable body sizes and foraging speeds might reveal whether visual identification of predator body shapes played some role in the similar responsiveness of aphids, or whether foraging speed and body size are the dominant cues. Although aphid spatial resolution is thought to be poor (Döring and Spaethe, 2009), visual cues may be integrated with vibrational cues in order to increase the accuracy of predator detection (Hartbauer, 2010, Gish, 2021). In my study, ladybird adults also encountered more aphids than lacewing larvae, in part because of their quicker pace around plants allowing for more exploration, which may account for the significantly higher raw number of drops and total defences they provoked in both aphid species.

Turning to the first part of Hypothesis 2, considering contact with a predator as another factor that aphids may use to assess risk, both aphid species were found to drop significantly more following contact with a predator than before contact. Further, most post-contact drops were initiated from adjacent to the predator, often immediately following contact. Clearly contact by a predator appears to act as a key trigger for dropping, the most extreme escape defence in terms of potential costs, as has been previously reported (Roitberg et al., 1979, Dill et al., 1990, Nelson and Rosenheim, 2006, Polin et al., 2014). It is unsurprising that contact with a predator would trigger immediate dropping – and therefore from close proximity – as it is an indicator of imminent predation threat. It is, however, interesting that aphids delay their dropping escape until such contact is made. I think this is unlikely to be due to aphids being unable to sense the presence of predators ahead of contact, as they would likely detect their movement through plant-borne vibrations as well as visually (Gish, 2021). Rather, I propose that aphids only drop post-contact because their predators often do not pose a direct danger and because the costs of dropping (compared to kicking or walking away) are high. In this study, predators rarely headed clearly towards a specific target aphid, rather they seemed to roam about plant sections without noticing that aphids were present until they incidentally contacted them. Most aphids, particularly pea aphids, also survived the first instances of contact with a predator and were able to react before being subdued, indicating that predators rarely made quick and decisive attacks on prey. It is worth noting here, that ladybirds have also been observed to ignore aphid prey when their locomotory activity has motivations other than searching for prey, such as searching for mates (Honěk, 1985). It may, therefore, be adaptive for aphids to wait until contact has been made before dropping to escape, as: 1) there is a high chance that the predator will not notice them or not show any interest in them, so they need not interrupt their feeding; 2) the first contact from a predator does not result in complete subjugation and so the aphid would likely have time to respond before the predator attacks; and 3) aphids benefit from avoiding the potentially substantial costs associated with dropping (Roitberg and Myers, 1979, Dill et al., 1990, Losey and Denno, 1998a, Nelson et al., 2004, Nelson and Rosenheim, 2006, Nelson, 2007, Agabiti et al., 2016). The importance of stimulus type and what contact with different stimuli might indicate to aphids is explored further in Chapter Six, where the post-dropping behaviour of aphids prompted to drop by the presence of live predators is compared to that of aphids prompted to drop by contact with a standardised, tactile stimulus.

Exploring the predator proximity aspect of Hypothesis 2 further, occasionally aphids did drop pre-contact, but these drops mostly still took place from the same plant section that the predator was exploring. It might make sense that aphids wait until a predator moves onto the same section and poses a direct threat so that potentially costly pre-contact dropping is warranted, but if making a pre-emptive drop then they should not wait until the predator is immediately beside them in case they are subdued. Alternatively, or additionally, regardless of whether or not a predator is in close proximity, if an aphid senses a change in vibration frequency that is sufficiently large to indicate risk, perhaps it will drop even if other triggers (i.e. predator contact) have not been elicited. Throughout this study, there were also a handful of drops by aphids that were not included in the analysis because they were not deemed to have been directly due to the movement or behaviour of the predator. Some of these for both pea and potato aphids were likely triggered by alarm pheromone detection as the drops coincided with periods when an aphid was being consumed or had a limb removed by a predator elsewhere on the plant. If alarm pheromone warns of predatory attack, dropping from a relatively long-distance may be adaptive where it enables aphids to escape a plant or area which has at least one predator present; the potential advantage this provides an aphid with, though, would depend on the behaviours exhibited post-dropping, explored further in Chapter Six. The fact that pheromone is released may be enough to raise the perceived threat levels such that the predator need not make contact or even be present on the aphid’s same plant section to trigger dropping. Other drops were noticed where pea aphids had recently dropped but then seemed to drop again as they were climbing back onto plants, but without any obvious further threat from the predator. If, as assumed, these drops were triggered more by aphids’ recent experience of threat and continuing stressed state rather than a new escape-inducing stimulus, it is unsurprising that many of these did not occur anywhere near the predator. Further excluded drops by both pea and potato aphids occurred for unknown reasons at relatively long distances from predators; perhaps these drops did not serve an antipredator function, rather they might have been triggered by a thermoregulatory need, or a desire to locate a different food source. Future studies with a greater ability to track all individuals and monitor microclimates and alarm pheromone presence might shed further light on the various triggers and functions of dropping.

Finally, by examining the defensive behaviour of the potato aphid as well as that of the pea aphid, my findings indicate that there are important interspecific differences. Although developmental stage, a factor which has been seen to influence the propensity to drop in aphids (Losey and Denno, 1998a, Gish and Inbar, 2006, Gish et al., 2012), was not controlled for across the species, the differences in relative use of different behaviours might reflect differences in their biology or life history. Pea aphids appeared to be more sensitive to the processes that trigger dropping, be they vibrations, visual cues, or contact with a predator. It is plausible that due to their different sizes, morphologies, detectability on host plants, and/or relative encounter rates with different natural enemies, pea and potato aphids have evolved different sensitivities to triggers of dropping that are adaptive to their own typical circumstances. For example, if these two species vary in their typical encounter rate with visual predators, this would influence which behaviours are most likely to be effective during predator-prey encounters. Some adult coccinellids do use vision as an important guide to their foraging (Nakamuta, 1984, Lim and Ben-Yakir, 2020) but different species vary in their dependence on visual cues (Harmon et al., 1998, Pervez and Yadav, 2018) and some species are known to continue their foraging activity overnight, during which they rely primarily on chemical cues (Norkute et al., 2020). If pea aphids are more likely to be detected visually by their most common natural enemies than potato aphids, and their predators are more likely to be successful in locating them on plants, then dropping might be a more essential component of their behavioural repertoire. However, further study into the relative sensitivity of aphids to triggers of dropping and their predators' prey detection abilities under varying abiotic conditions is needed to understand interspecific behavioural differences more fully. Additional experiments utilising aphid lines freshly sourced from the wild would also be valuable in confirming how closely the antipredator behaviours observed in this study reflect those shown by aphids of either species when they are not reared in the absence of natural predators. Long-term culturing of aphids has previously been found to reduce some natural responses (Thieme and Dixon, 2015).

In conclusion, dropping appears to be a defence deployed only when the risk of imminent predation is assessed as being high, where factors that might increase the propensity of aphids to drop include: faster-foraging predators such as adult ladybirds, predator proximity, and contact with a predator that may trigger more targeted, intensive search by the predator. As discussed in Chapter Three, dropping can carry fitness costs (Roitberg et al., 1979, Dill et al., 1990, Nelson, 2007, Agabiti et al., 2016), and so flexible use of it as an extreme defence only when the predation risk is assessed as being high would be adaptive. Future research building on what is already known about post-dropping tonic immobility (Niku, 1975, Wohlers, 1981, Bilska et al., 2018) and aphid return onto plants post-dropping (Niku, 1975, Niku, 1976, Roitberg et al., 1979, Gish and Inbar, 2006) will help to develop an even fuller picture of the trade-offs involved with dropping. Indeed, in Chapter Six I explore some key post-dropping behaviours shown by pea and potato aphids, including rate of recovery back to plants and tonic immobility. Additionally, factors relating to the predatory threat that might influence dropping likelihood also warrant more detailed investigation, for example predator approach trajectory. Several aphid species have been seen to walk away from predators (instead of dropping) more frequently when approached from the front rather than from the rear (Dixon, 1958, Hajek and Dahlsten, 1987). Other aphids may also be less likely to drop from host plants if they have a clear visual warning of an approaching predator and time to walk away pre-contact. This idea is picked-up on again in Chapter Seven, where I discuss my fieldwork looking into both ladybird searching behaviour and aphid antipredator responses. Studying the impact of multiple predator types in a single predation situation would also prove valuable. More broadly, future research into dropping behaviour – or indeed any other antipredator defence – in any prey species may benefit from investigating the use of a specific defence within the context of the available antipredator portfolio. Understanding the decision-making that might drive the use of one defence over alternatives is key to shedding light on the various costs and benefits of all defences and the selective pressures that could have led to their development over evolutionary time.

# Chapter 6: Post-dropping behaviour of potato aphids (*Macrosiphum euphorbiae*)

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Dropping behaviour is an effective antipredator defence utilized by many insects including aphids, which drop from plants to lower plant parts or underlying substrates to avoid attack from predatory invertebrates. While research commonly focusses on triggers of dropping, less attention is given to what happens to prey individuals following escape drops. This chapter reports findings from additional data collected on potato aphids (*Macrosiphum euphorbiae*) during the experiment described in Chapter 4. In this chapter, the duration of tonic immobility, recovery rates, and cases of 'instant recovery' (re-clinging to lower plant parts) exhibited by potato aphids that dropped from young potted plants in response to introduced ladybird (*Adalia bipunctata*) adults, lacewing (*Chrysoperla carnea*) larvae, and a standardised tactile stimulus were investigated in relation to a range of environmental factors. Air temperature had a negative correlation with the duration of post-dropping tonic immobility; as temperature increased, time spent motionless decreased. Aphids also showed a pattern of increased recovery rate at higher temperatures. Aphids may be selected to move off the substrate quicker to avoid risks of overheating/desiccation at higher temperatures; and/or higher body temperature facilitates locomotion. Stimulus type also influenced recovery rate back to the original plant, with aphids generally recovering after the standardised stimulus quicker than after dropping triggered by a real predator. Considering cases of instant recovery onto lower-reaches of the host plant, plant height influenced the likelihood of re-clinging, with aphids that managed to instantly recover dropping from, on average, taller plants than aphids that dropped to the substrate. Plant architecture could mitigate the costs of dropping for aphids, but further studies quantifying understory foliage cover are needed.

## 6.1 Introduction

In Chapter Three, I described dropping as an important antipredator behaviour, wherein a prey individual releases itself from a substrate (or ceases powered flight), passively or actively, such that gravity, wind or water currents power its subsequent escape from a perceived threat. Although a taxonomically widespread defense, dropping by insects has received by far the most attention, as discussed in Chapter Three. But while considerable research efforts have developed understanding of on-plant triggers of dropping in insects, the behaviours individuals exhibit afterwards are comparatively neglected. When an insect drops from a plant onto the underlying substrate to avoid predation, it is exposed to many costs and risks associated with being off the plant, such as: lost feeding time, risk of desiccation, and potential exposure to new predators (Losey and Denno, 1998a, 1998b, Nelson, 2007, Agabiti et al., 2016). Yet, previous studies have observed insects behaving in ways that prolong the time spent off plants after dropping (see later). This exploratory study aims to investigate underexplored links between dropping and post-dropping behaviours.

Tonic immobility (hereafter referred to as TI) is one behaviour that animals can exhibit post-dropping. TI describes the unlearned adoption of a motionless posture (sometimes a posture that resembles a dead individual, hence the alternative term ‘death-feigning’) by prey individuals that is triggered by the presence of a perceived threat (Ruxton et al., 2018). It has been described – often anecdotally – across wide-ranging taxa, including vertebrates (see Cassill et al. [2008] for a partial list and references, and the following recent examples: Sánchez-Paniagua and Abarca [2016], Freret-Meurer et al. [2017], Golubović et al. [2021]) and invertebrates (see Cassill et al. [2008] for a partial list and references, and the following recent examples: Segovia et al. [2018], Wilkie [2019], Lira et al. [2020], Oyen et al. [2021]), but most quantitative accounts of the phenomenon concern insects (Miyatake et al., 2004, Ohno and Miyatake, 2007, Miyatake et al., 2009, Nakayama and Miyatake, 2010a, Ritter et al., 2016, Neves and Pie, 2018, Uchiyama et al., 2019). In insects, decreased ventilatory movements and the maintenance of a stiff posture are common during TI (Rogers and Simpson, 2014); the adaptive function of this for individuals following an escape drop in response to a foliar-foraging predator may be reduced localization by other predators, such as birds or ground-foraging beetles that have detected the dropping movement (Wohlers, 1981, Miyatake et al., 2009). A few studies have found that the duration of TI in dropped insects can vary depending on a range of factors, including the nature of the stimulus that triggered dropping (Wohlers 1981), height dropped from (Niku, 1975), and ambient temperature (Miyatake et al., 2008). But relationships between the frequency or duration of tonic immobility and environmental factors such as temperature, for example, are not always consistent across insect groups (Ritter et al., 2016) and much remains unexplored.

Following TI, normal behaviour can rapidly resume (Rogers and Simpson 2014), but the mode of recovery of dropped insects back to host plants shows variation. For example, some aphids (family Aphididae) undertake different characteristic movements after post-drop TI: individuals either run slowly but show a high turning rate (deemed ‘searchers’) or run quickly but show a low turning rate (deemed ‘runners’) (Niku, 1975, Niku, 1976, Roitberg et al., 1979, Ben-Ari et al., 2015). A limited number of studies exploring post-drop recovery suggest that the nature of the stimulus that prompted dropping may influence the type of movement shown during recovery (Phelan et al., 1976, Wohlers, 1981), but this requires further study and the influence of other factors on the rate at which dropped insects return to plants is poorly understood. For any insect that has dropped to the substrate from a plant, the nature of any TI and recovery behaviours it exhibits will have important consequences for the costs it experiences, its survival likelihood, and overall fitness.

Of course, the most effective way of mitigating the costs of dropping is to avoid falling all the way to the substrate in the first instance. As discussed in Chapter Three, many flightless insects exhibit an ‘aerial righting’ reflex, wherein they change their body posture in such a way - after releasing from a substrate - that their ventral side and any adhesive organs are directed downwards (Yanoviak et al., 2009, Jusufi et al., 2011, Ribak et al., 2013, Meresman et al., 2014, Zeng et al., 2017, Kane et al., 2021). This enables re-grasping of plant material underlying the position they dropped from, so that they do not fall to the ground after dropping (Nelson, 2007, Ribak et al., 2013, Meresman et al., 2017); I also refer to this as ‘instant recovery’ back to the host plant. Individuals’ reaction to detachment from plants and aerial righting take time, and so it is unsurprising that the vertical position on the plant from which an insect drops appears to influence the likelihood of re-clinging (Ribak et al., 2013, Meresman et al., 2017). Additionally, several characteristics of host plants have been found to relate to the success of dropped insects in re-clinging, including leaf architecture (Matsubara and Sugiura, 2018), host plant growth form (Matsubara and Sugiura, 2021), foliage density, and full plant height (Meresman et al., 2017), but the link between dropping and this important cost-mitigating behaviour remains underexplored.

This present chapter aims to highlight often-underappreciated post-dropping behaviour as an important aspect of predator-prey interactions. Potato aphids, my study species, are one of many commercially-important aphid species that use dropping as a significant component of their antipredator behavioural repertoire, as demonstrated in the findings of Chapters Four and Five. They can colonize potato plants at an early developmental stage, and the colony can go through a number of generations during the life cycle of the plant (AHDB, 2015). Here, I explored potato aphids' post-dropping behaviour (TI, recovery, and re-clinging ability or 'instant recovery') using additional data collected during the study that was described in detail in Chapter Four and also formed the basis of Chapter Five. Potato aphids are my focal species here as more post-dropping data was collected for this species compared to pea aphids (*Acyrthosiphon pisum*) during my experiments. In examining TI, my interest was its duration rather the specific form exhibited. When considering recovery, I did not focus on the mechanism of recovery (i.e., the movements or walking routes of aphids), rather on time taken. These design decisions were taken because I am interested in investigating dropping from a behavioural-ecology framework (instead of its mechanics), by considering the benefits - i.e. survival – against the time spent off-plant, which can be presumed to correlate with associated costs; such as lost feeding time, risk of desiccation, and time exposed to other potential risks on the substrate (Losey and Denno, 1998a, 1998b, Nelson, 2007, Agabiti et al., 2016). Potato aphids' TI and recovery rates were opportunistically explored in relation to a range of different factors: stimulus type; substrate; maximum height of plants; distance dropped from original host plant; and ambient temperature. Some of these variables have been previously explored in other insect species, but not potato aphids; and no study in any species has systematically explored them in concert. The ability of potato aphids to re-grasp underlying plant material after dropping was explored in relation to several plant-related factors: the position on plants that aphids dropped from (that is, the type of plant section an aphid was located on, e.g. stem, petiole, leaf upperside, leaf underside); plant age; and the maximum height of plants (similar to the 'field height' measure used by Meresman et al., 2017). Because this exploratory study considers many factors for each post-dropping behaviour, I detail my specific predictions and the rationale behind them in Table 6.1.

**Table 6. 1:** Summary table of predictions for the study.

| Post-dropping behaviour | Environmental factor | Prediction | Rationale |
|---|---|---|---|
| ***Tonic immobility (TI)*** | Stimulus type | TI duration will increase following aphid drops in response to a standardised tactile stimulus, compared to drops in response to a predator. | Previously reported in pea aphid (Wohlers, 1981). The significance of the different time aphids spend in TI is unknown (Wohlers, 1981) but, contact may indicate greater risk of imminent predation than other cues. Although predators could potentially contact prey in this study, contact is guaranteed with the standardised stimulus while drops may occur in response to visual and/or vibrational cues when facing predators. |
| | Substrate | TI duration will be shorter following drops onto sand compared to drops onto soil. | Aphids are vulnerable to desiccation (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979). Sand retains less moisture than the sand–perlite–peat compost mix I refer to as ‘soil’ throughout, and therefore might represent a greater risk of desiccation. |
| | Maximum height of plants | TI duration will not be influenced by the maximum height of plants. | Niku (1975) found that the time pea aphids spend in TI negatively correlated with the height of the fall, in a study that experimentally manipulated drop heights from 10-100 cm. Roitberg et al. (1979) did not find this correlation in pea aphids, but their study included unmanipulated height differences spanning only 10-25 cm. The maximum height of the young plants used in this current study was not experimentally-manipulated and constituted only a narrow range of relatively short heights, closer to those of Roitberg et al. (1979). |

| | | | |
|---|---|---|---|
| | Horizontal distance dropped from original plant | TI duration will decrease the greater the horizontal distance dropped from the original plant. | Dropping a greater horizontal distance from the original host means that returning to that plant will unavoidably take longer, increasing exposure to the risks associated with being off-plant (e.g. Losey and Denno, 1998a, 1998b, Nelson, 2007, Agabiti et al., 2016). |
| | Temperature | TI duration will decrease with higher ambient temperatures. | As air temperature increases, higher body temperatures might facilitate greater locomotion, as seen in some beetles (Miyatake et al., 2008), but also increase risk of desiccation for aphids (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979). |
| ***Recovery rate*** | Stimulus type | Recovery rate will be slower following drops in response to predators, compared to drops in response to astandardised stimulus. | Dropped aphids of various species that have encountered foraging predators or detected alarm pheromone prior to dropping show a tendency to travel greater distances before recovering to plants (‘runners’), while aphids dropping in response to touch show more immediate ‘searching’ behaviour to aid rapid return to hosts (Niku, 1975, Phelan et al., 1976, Wohlers, 1981). |
| | Substrate | Recovery rate will be slower across sand than across soil. | Dropped pea aphids have been reported to frequently change course during their return to plants after encountering difficulties on rough, dry or loose terrain (Roitberg et al., 1979). Sand is looser than the sand–perlite–peat compost mix I refer to as ‘soil’ throughout, and might make locomotion more challenging compared to a more tightly-compacted substrate, slowing recovery. |
| | Maximum height of plants | Recovery rate will not be influenced by the maximum height of plants. | The maximum height of the young plants used in this study was not experimentally-manipulated and constituted only a narrow range of relatively short heights. |

| | | | |
|---|---|---|---|
| | Horizontal distance dropped from original plant | Recovery rate will increase the greater the horizontal distance dropped from the original plant. | As that for TI. |
| | Temperature | Recovery rate will increase with higher ambient temperatures. | As that for TI. |
| ***Re-clinging (‘Instant recovery’)*** | Plant positions dropped from | Instant recovery will occur more frequently following drops from plant positions that are more likely to overhang other plant structures, e.g. leaf undersides. | Aphids dropping from positions that are more likely to overhang other plant structures are more likely to encounter underlying plant material that they can re-cling to. |
| | Plant age | Instant recovery will occur more frequently following drops from older plants. | Pea aphids’ ability to re-cling to plant material when dropping positively correlates with plants’ foliage cover (Meresman et al., 2017). Older plants, which have presumably grown larger and represent a more dense foliage cover, may present dropping aphids with more underlying plant material on which to re-cling. |
| | Maximum height of plants | Instant recovery will occur more frequently following drops from plants with greater maximum heights. | Pea aphid post-dropping instant recovery has been found to occur more commonly on taller plants; the chances of falling from objectively higher starting heights (during which there is more time for aerial righting) is greater on relatively taller than shorter plants (Meresman et al., 2017). |

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|---|---|---|
|  |  | Additionally, pea aphids’ ability to re-cling to plant material when dropping is positively dependent on plants’ foliage cover (Meresman et al., 2017). Taller plants have presumably grown more outwards as well as upwards, and therefore might present a greater underlying foliage cover for dropped aphids to encounter and re-cling to. |

## 6.2 Materials and methods

The data collection for this chapter took place at the James Hutton Institute (Dundee, UK), alongside the experiment described in Chapter Four. As such, information concerning the growth of potato plants and the rearing and maintenance of potato aphids (as well as the predators used) is detailed in Chapter Four. The post-dropping behaviours of 8-9 d old 3rd-4th instar potato aphids were recorded following encounters with lacewing larvae and adult ladybirds on live, potted, immature potato plants 2-3 weeks from planting. Data collection occurred across three parts, detailed below.

### 6.2.1 Part 1: Predator-induced drops

The experimental protocol set out in Chapter Four constituted Part 1 of data collection, with several important additional steps being included in the methodology for the purposes of this chapter. Firstly, when preparing the pots containing two immature potato plants pre-infestation, half of the pots in the experiment were left with the sand–perlite–peat compost mix (referred to as ‘soil’ throughout) as the substrate and half had a thin top layer of fine white Scalare river sand (grains <0.5mm) added, determined randomly for any given assay. Further, the maximum height of the immature plants in any given pot was recorded to the nearest 0.5 cm, as well as the plants’ age since potting (in days).

At the start of each assay, the temperature (to within 0.5°C) was recorded using a Brannan (Cumbria, UK) ‘Push button Minimum/Maximum’ thermometer, immediately before the first predator was introduced during filming. Assays were then conducted as described in Chapter Four, with the predator’s foraging period being filmed from overhead with a camcorder fixed to a tripod. As the predator moved about the young potato plants, details of any dropping behaviour exhibited by aphids apparently in response to the predator’s presence were dictated to the camera by the experimenter; this included drops from the predator’s current plant section and on nearby sections. When an aphid dropped, the following additional metrics were recorded, if observed: which predator type triggered the drop, what plant position the aphid dropped from (considered as stem, petiole or petiolule or lateral stem, leaf upperside, or leaf underside), the time the aphid dropped, the horizontal distance the aphid landed from the stem of its original host plant (to the nearest 0.1 cm, measured either with a flexible paper ruler during trials or calculated from the planar view of recordings post-trials), the time the aphid moved again after any period of TI, and the time when the aphid subsequently had all its legs back onto a plant following recovery. Any cases of aphids re-clinging to underlying plant parts after dropping, rather than falling to the substrate, were also observed and their occurrence dictated to the camera by the experimenter. Once the 30-minute filmed period of predator foraging described in Chapter Four was completed the predator was removed, ending Part 1 of data collection.

### 6.2.2 Parts 2 and 3: Standardised stimulus-induced drops

To further explore the post-dropping tendencies of potato aphids, two additional parts followed Part 1 of assays. Following the removal of the predator, filming continued and temperature was recorded for a second time. For Part 2 of trials, then, each individual aphid remaining on the plants – that is those that had either not left their original host plant during the predator foraging period, or those that had left (either via walking or dropping) but had then re-settled on one of the plants prior to the end of Part 1 – was subjected to an initial bout of standardised mechanical stimulus. The standardised stimulus consisted of a brief and gentle stroke from a small, fine paintbrush (similar to Wohlers, 1981) running from head-to-tail and just making contact with the individual. If any aphids dropped in response to this contact, the relevant metrics listed above were recorded, so long as the associated behaviours occurred within 5 minutes of the standardised stimulus (after this time the recording was stopped). After Part 2, pots were left for 30 min in order to allow the aphids to recover. After this time, the video camera was set to record again, and the temperature was recorded for a final time. Part 3 then consisted of a second bout of standardised stimulus and recording of dropping responses and post-dropping behaviours, carried out on any aphids that were still settled or had re-settled on plants in the same way as in Part 2.

A total of 32 assays (each with three parts) took place, 16 with each predator type in Part 1. For all three parts to the post-dropping experiment, Behavioural Observation Research Interactive Software (BORIS) (Friard and Gamba, 2016) was used to watch the video footage, code behaviours, record their timings, and measure some distances aphids dropped from their original host plant. From the time and distance measures, post-dropping TI durations and (only where aphids returned to their original host plants) recovery rates (cm/s) were calculated.

### 6.2.3 Analysis

All statistical analyses were conducted using R version 4.0.3 (R Core Team, 2020).

## 6.3 Results

### 6.3.1 Post-drop tonic immobility (TI) duration

Data from Part 1 of the experiment, during the predator foraging period, were used to explore whether different variables affect the duration of TI potato aphids undergo post-dropping. Only drops where either a ladybird adult or lacewing larva was considered to be the stimulus were included, as the focus of the chapter is behaviour following antipredator dropping. The only exception to this was when stimulus type more broadly – rather than predator type alone – was considered and, in this case, data from Parts 2 and 3 of the experiment were also utilized. For all analyses concerning TI, cases of instant recovery and cases where aphids fell off plant pots entirely were excluded.

#### *6.3.1.1 Stimulus type*

During Part 1 of trials, the duration of TI was recorded for 48 of the 51 cases where ladybird adults appeared to trigger potato aphids to drop onto the underlying substrate. TI durations were recorded for all 22 of the cases where lacewing larvae caused aphid drops to the substrate. Comparing the mean durations of TI, no significant difference was found between aphids that dropped in response to ladybirds (mean ± standard deviation [hereafter sd]: 11.5 ± 25.1 s) and those that dropped in response to lacewings (mean ± sd: 4.7 ± 5.1 s) (Welch two-sample t-test: t = 1.8, df = 54.9, P = 0.078). The same was true in a comparison of median TI duration (median ± interquartile range [hereafter IQR] for ladybird-induced drops: 2.1 ± 4.6 s, median ± IQR for lacewing-induced drops: 2.8 ± 5.0 s, Wilcox test W= 549, P = 0.80). Parts 2 and 3 of trials were then considered in order to test whether the duration of TI differed significantly depending on whether aphids were faced with natural predators or standardised stimuli. Both predator types were combined for this analysis, due to the lack of significant difference in TI duration reported above, and compared with the TI durations recorded following drops caused by the first standardised stimulus (immediately after the predator foraging period in Part 1) and the second standardised stimulus (following a 30-minute recovery period after Part 2) separately. Post-drop TI durations were recorded for: 70 of the 73 times aphids dropped in response to predators, 108 of the 111 times aphids dropped in response to the first standardised stimulus, and 89 of the 92 times aphids dropped in response to the second standardised stimulus. A one-way ANOVA found no significant difference in the mean duration of TI of aphids dropping as a result of the different stimuli (mean ± sd for predator: 9.3 ± 21.1 s, first standardised stimulus: 6.7 ± 12.4 s, second standardised stimulus: 14.5 ± 43.2 s, F = 1.69, num df = 2.00, denom df = 129, P = 0.19). Similarly, a Kruskal-Wallis rank sum test found no significant difference in the median durations either (medians ± IQR for predator: 2.1 ± 4.8 s, first standardised stimulus: 2.3 ± 6.7 s, second standardised stimulus: 2.6 ± 7.8s, Kruskal-Wallis chi-squared = 0.23, df = 2, P = 0.892). Any such comparisons must be interpreted cautiously, since individuals were of course not randomized to stimulus type, and thus confounding factors may be important.

#### *6.3.1.2 Substrate*

TI duration was recorded for 35 of the 37 of the predator-induced drops that resulted in aphids landing on sand as the underlying substrate, and 35 of the 36 cases where aphids landed on soil. A Welch two-sample t-test found no significant difference (t = 1.28, df = 41.0, P = 0.21) in the mean duration of TI of dropped aphids on sand (mean ± sd: 12.5 ± 28.3 s) compared to those that dropped on soil (mean ± sd: 6.1 ± 9.1 s). Similarly, a Wilcox test found that there was also no significant difference (W = 468, P = 0.09) in median duration of TI of dropped aphids on sand (median ± IQR: 1.4 ± 4.5 s) compared to those that dropped on soil (median ± IQR: 3.3 ± 5.5 s).

#### *6.3.1.3 Maximum height of plants*

Previous studies have reported contrasting findings that height dropped from does (Niku, 1975) and does not influence aphid TI duration (Roitberg et al., 1979), and so height was explored further here in another context where plant height was not experimentally manipulated. The relationship between the height of plants and the duration of aphid TI post-dropping was explored for the 70 cases where TI duration was recorded (73 drops were observed across Part 1). The maximum height of the immature plants ranged from 9.0 - 30.0 cm (mean: 15.1 cm, median: 11.0 cm), and TI durations ranged from 0.4 - 133.9 s (mean: 9.3 s, median: 2.1 s). A Pearson's product-moment correlation test found no significant correlation between maximum height of plants and aphids’ post-drop TI durations (t = 0.08, df = 68, P = 0.94).

### 6.3.1.4 Horizontal distance dropped from original plant

The relationship between the horizontal distance dropped aphids landed from their original host plant and the duration of their subsequent TI was explored for the 67 cases where both the distance and TI duration were recorded. The distance landed from original host plant stems ranged from 0.2 - 9.0 cm (mean: 3.0 cm, median: 3.0 cm), and TI durations ranged from 0.4 - 134 s (mean: 9.7 s, median: 2.4 s). A Pearson's product-moment correlation test found no significant correlation between distance landed from original host plant and subsequent TI durations (t = -0.21, df = 65, P = 0.84).

### 6.3.1.5 Temperature

The relationship between the glasshouse temperature at the start of Part 1 and the duration of aphid TI post-dropping was explored for the 70 cases where TI duration was recorded. Temperatures ranged from 22 - 40°C (mean: 29.9°C, median: 29.5°C); descriptive statistics for TI durations are given in section 6.3.1.3 above. A Pearson's product-moment correlation test found a significant negative correlation between the temperature recorded at the beginning of the predator foraging period and the duration of aphids' TI post-dropping (t = -2.2, df = 68, P <0.05). At higher glasshouse temperatures, aphids appeared to spend less time motionless on the substrate (Figure 6.1).

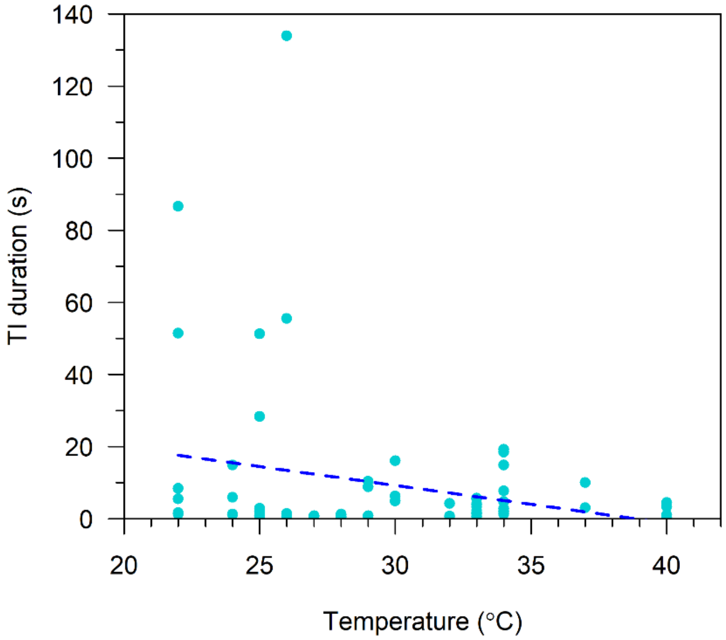


**Figure 6. 1:** As the glasshouse temperature recorded at the beginning of Part 1 increased, the duration of dropped potato aphids' tonic immobility (TI) on the underlying substrate appeared to decrease (n = 70).

### 6.3.2 Post-drop recovery rates back to original host plants

The recovery of aphids considered in these analyses refers to activity occurring subsequent to any period of TI recorded post-dropping. The rate of recovery (cm/s) was calculated for all aphids that returned to their original plant after landing on the substrate, using the time they started moving again after any TI, the time they had all their feet back on their original plant, and the approximate horizontal distance they landed at from the stem of their original plant. Importantly, the path the aphid followed -whether it was straight or torturous - was not recorded. Therefore, while distance was measured as a straight line and rate of recovery was calculated as distance over time, this does not guarantee that the aphid moved directly back to its original host plant. An aphid may have moved quickly across the substrate, but looped around on the substrate before its return, so although its speed across the substrate might have been great its rate of recovery may have been relatively slow. I focus on recovery rate as an informative measure regarding the relative time and energy costs I can assume aphids suffer from being off plants and not feeding. As with the TI analyses, data from exclusively Part 1 of the experiment was used to explore whether different variables affect aphid recovery rate post-dropping, with the exception of the broader stimulus analyses; for which, data from Parts 2 and 3 of the experiment were also utilized. Also, cases of instant recovery and cases where aphids fell off plant pots entirely were again excluded.

#### *6.3.2.1 Stimulus type*

For Part 1, the rate of recovery was calculated for all 23 instances where aphids dropped in response to a foraging ladybird adult, and for eight of the nine instances where aphids dropped in response to a foraging lacewing larva. No significant difference was found (Welch two-sample t-test: t = - 1.06, df = 8.6, P = 0.32) between the mean (mean ± sd for ladybird-induced: 0.071 ± 0.060 cm/s, lacewing-induced: 0.114 ± 0.107 cm/s) or median (median ± IQR for ladybird-induced: 0.063 ± 0.065 cm/s, lacewing-induced: 0.101 ± 0.167 cm/s, Wilcox test W = 76, P = 0.49) recovery rate of aphids dropping in response to the different predatory stimuli.

Parts 2 and 3 of trials were then considered to explore whether aphids' recovery rate back to their original host plants significantly differed depending on whether dropping had been provoked by a foraging predator (as with the TI analyses, data for ladybird adults and lacewing larvae were combined), a first standardised stimulus (Part 2), or a second standardised stimulus (Part 3). Subsequent recovery rates (cm/s) were calculated for 31 of the 32 drops in response to predators that were followed by a return to the original host plant, 24 of the 25 drops in response to the first standardised stimulus that were followed by a return to the original host plant, and all 25 drops in response to the second standardised stimulus that were followed by a return to the original host plant. A Kruskal-Wallis rank sum test found a significant difference in the median recovery rate of aphids dropping in response to different stimuli (median ± IQR for predator: 0.068 ± 0.078 cm/s, first standardised stimulus: 0.121 ± 0.085 cm/s, second standardised stimulus: 0.123 ± 0.108 cm/s, Kruskal-Wallis chi-squared = 9.30, df = 2, P <0.01). Additionally, a one-way ANOVA found a significant difference in the mean recovery rate of aphids dropping in response to different stimuli (mean ± sd for predator: 0.082 ± 0.075 cm/s, first standardised stimulus: 0.141 ± 0.081 cm/s, second standardised stimulus: 0.141 ± 0.124 cm/s, F = 4.57, num df = 2.0, denom df = 45.6, P <0.05). A post-hoc Games-Howell test (suitable for unequal variances) then found a significant difference between recovery rate following drops triggered by predators and drops triggered by the first standardised stimuli (P <0.05). Potato aphids appeared to recover back to their original host plant at a slower rate when their drop from that plant had been provoked by the presence of a natural predator, compared to when their drop had been triggered by contact from the standardised stimulus (Figure 6.2).

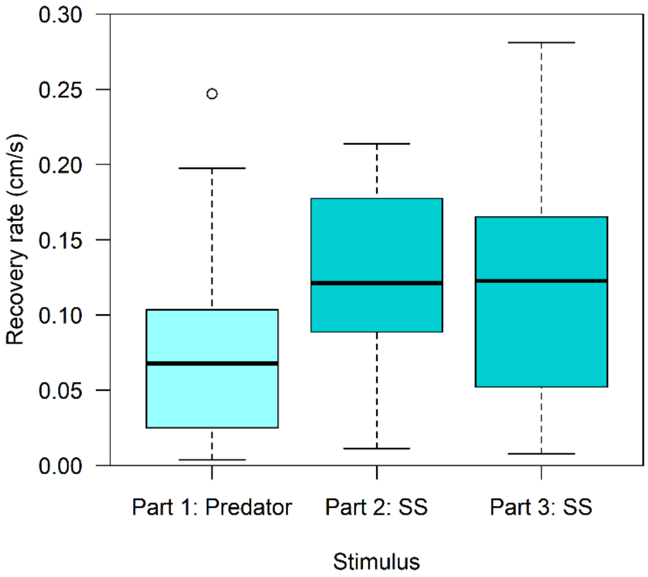


**Figure 6. 2:** The recovery rates (cm/s) of potato aphids (excluding any periods of tonic immobility) that recovered back to their original host plant, following: drops provoked by natural predators (ladybird adults and lacewing larvae) in Part 1 (n = 31), drops provoked by the first bought of standardised stimulus (SS) in Part 2 (n = 24), and drops provoked by the second bought of SS in Part 3 (n = 25). The SS consisted of a brief and gentle head-to-tail stroke from a fine paintbrush.

#### *6.3.2.2 Substrate*

Recovery rate was recorded for all 13 cases in Part 1 where aphids recovered back to their original plants after dropping to sand as the underlying substrate, but for only 18 of the 19 cases where the underlying substrate was soil. No significant difference (Welch two-sample t-test: t = -1.57, df = 28.03, P = 0.129) was found in the mean (mean ± sd for sand: 0.060 ± 0.051 cm/s, soil: 0.099 ± 0.087 cm/s) or median (median ± IQR for sand: 0.041 ± 0.061 cm/s, soil: 0.072 ± 0.115 cm/s, Wilcox test W = 92, P = 0.332) recovery rate of aphids dropping onto the different substrates.

#### *6.3.2.3 Maximum height of plants*

The relationship between the height of the immature plants used in this study and the recovery rate of aphids was explored for the 31 cases where aphids returned to their original host plants following antipredator dropping and all necessary time and distance measures were recorded (32 drops where aphids returned to their original host plants were observed in total across Part 1). The maximum height of plants in these trials ranged from 9.0 - 25.0 cm (mean: 13.7 cm, median: 11.0 cm), and recovery rates ranged from 0.004 – 0.305 cm/s (mean: 0.082 cm/s, median: 0.068 cm/s). A Pearson's product-moment correlation test found no significant correlation between maximum height of plants and aphids’ post-drop recovery rates back to their original host plants (t = -1.02, df = 29, P = 0.31).

#### *6.3.2.4 Horizontal distance dropped from original plant*

The relationship between the horizontal distance dropped aphids landed from their original host plant and the rate of their recovery back to that plant was explored for the 31 cases where all necessary time and distance measures were recorded. The distance landed from host plant stems ranged from 0.2 – 6.0 cm (mean: 2.4 cm, median: 2.5 cm); descriptive statistics for recovery rates are given in section 6.3.2.3. A Pearson's product-moment correlation test found no significant correlation between straight horizontal distance landed from original host plant and subsequent rate of recovery (t = 0.15, df = 29, P = 0.88).

#### *6.3.2.5 Temperature*

The relationship between the glasshouse temperature at the start of Part 1 and the recovery rate of aphids was explored for the 31 cases where aphids returned to their original host plants following antipredator dropping and recovery rate could be calculated. Temperatures ranged from 22 - 40°C (mean: 30.5°C, median: 33.0°C); descriptive statistics for recovery rates are given above. A Pearson's product-moment correlation test found a significant positive correlation between the temperature recorded at the beginning of the predator foraging period and the rate of aphids' recovery back to their original host plants (t = 2.60, df = 29, P <0.05). At higher glasshouse temperatures, aphids appeared to return to plants at a relatively faster rate (Figure 6.3).

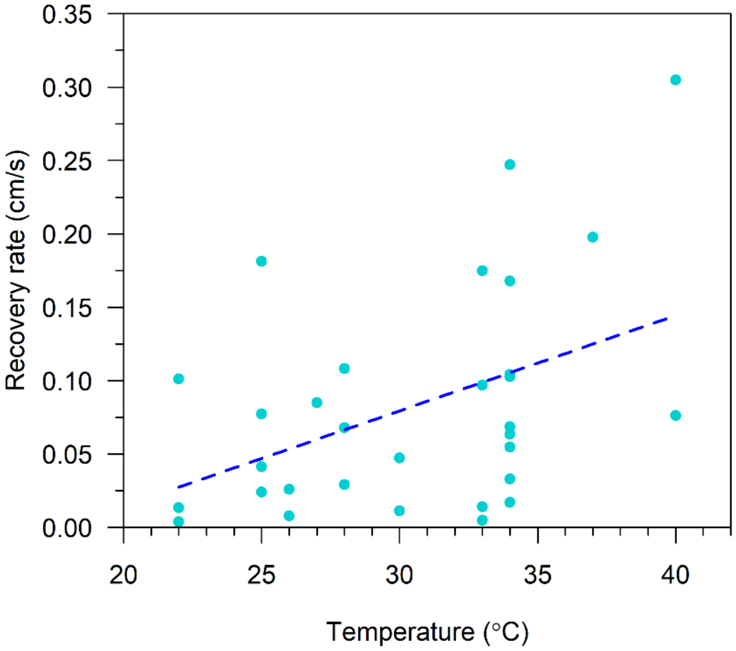


**Figure 6. 3:** As the glasshouse temperature recorded at the beginning of Part 1 increased, the post-drop rate of recovery of potato aphids that recovered back to their original host plants increased (n = 31).

## 6.3.3 Mitigation of dropping/re-clinging/instant recovery

Here I consider cases where aphids landed on an underlying section of their current plant following a drop (what I deem instant recovery) across all three parts of the experiment – this may be one means by which aphids can mitigate the costs associated with dropping. 31 cases of instant recovery occurred in total, with 7 following drops triggered by predator contact (6 by ladybird adults and 1 by a lacewing larva) and 24 following drops triggered by the standardised stimulus (11 by the first standardised stimulus and 13 by the second).

### *6.3.3.1 Plant positions dropped from*

Thirty of the 31 observed cases of instant recovery followed drops from leaf undersides, with the other one occurring following a drop from a plant's main stem.

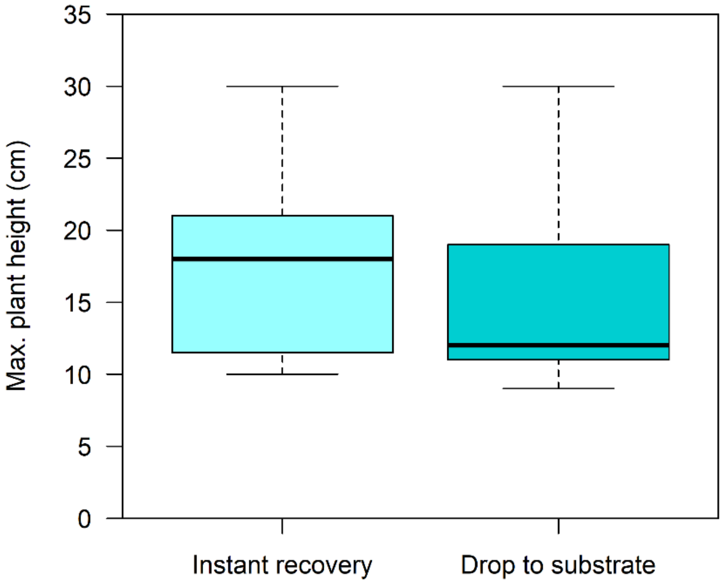


**Figure 6. 4:** The maximum height of plants in pots on which cases of instant recovery (where aphids landed on an underlying section of their current plant following a drop) occurred (n = 31), and the maximum height of plants in pots on which aphids dropped to the substrate (n = 313) across all three parts of the experiment. The maximum plant height for any given trial occurred in the data the same number of times that instant recovery or dropping to the substrate occurred during that trial. Instant recovery appeared to occur more frequently following drops from plants with a greater maximum height compared to the maximum heights of plants from which dropping to the substrate occurred.

#### *6.3.3.2 Plant age and maximum height of plants*

Using data from all 31 cases of instant recovery, a chi-squared test for given probabilities found no significant difference between the proportion of instant recovery following drops and the proportion of total drops that occurred on experimental pots with plants of different ages ($\chi^2_8 = 0.28$, P = 1). The immature plants in this experiment, however, only ranged from 18 - 26 d old and their age did not consistently reflect relative heights or foliage cover. To explore another plant factor, then, the maximum heights of plants in pots on which instant recovery occurred (range: 10.0 – 30.0 cm, mean: 17.4 cm, median: 18.0 cm) were then compared with the maximum heights of plants in pots across cases of dropping where instant recovery did not occur and aphids fell to the underlying substrate (range: 9.0 – 30.0 cm, mean: 15.02 cm, median: 12.0 cm). Because cases of dropping occurred in all trials, all of the plants on which instant recovery occurred appeared in both groups (this is seen in the considerable overlap of value ranges in Figure 6.4). But consideration of the maximum heights of plants in pots on which no instant recovery occurred and the relative frequency of cases of instant recovery or drops to the substrate on any individual pair of plants could still be informative, despite the measure being crude. For this reason, the maximum plant height for each given trial was repeated in the data for the number of times that instant recovery or dropping to the substrate occurred, so there were a total of 31 heights listed for the instant recovery data and 313 heights for the drops to the substrate data. No significant difference was found between the medians, though the p-value was verging on significance (Wilcox test W = 5876, P = 0.05), but a Welch two-sample t-test found a significant difference between the means (t = 2.15, df = 35.8, P <0.05). The mean maximum height of plants in pots on which instant recovery was seen to occur following drops was significantly greater than that of plants in pots on which instant recovery did not occur following dropping.

## 6.4 Discussion

Overall, potato aphids were found to vary in their post-dropping behaviour, indicating that these behaviours occur flexibly under different conditions; they are an important aspect of predator-prey interactions where dropping is an option within aphids' defensive repertoires. Over the course of the 32 assays, there were 17 instances of aphids being grabbed and consumed by foraging predators while on plants; no aphids that dropped to the substrate were ever pursued by the predator that triggered the drop. Although I explored the influence of a range of variables on post-dropping TI and recovery rates, it is unlikely that the significant results I detected were due to type I error. The effects I detected were always in the predicted directions and the associated p-values were never marginal. To the best of my knowledge, this chapter provides novel findings regarding the significant influence of several factors on potato aphids' TI and recovery rates post-dropping, as well as the first data collected on influencers of their re-clinging abilities.

### 6.4.1 Post-drop tonic immobility (TI) duration

A striking aspect of post-dropping behaviour was that droppers often showed a period of stillness (akin to TI) prior to moving across the substrate to recover to a plant. As predicted in Table 6.1, temperature had a significant negative correlation with the duration for which potato aphids exhibited TI after dropping. As the underlying substrate gets hotter, the risk of overheating and desiccation increases for aphids (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979, Dill et al., 1990), and therefore dropped aphids may be selected to reduce the time spent in an immobile state and increase activity that will result in escape from the substrate. Further, seed beetles have been shown to exhibit TI less strongly at higher ambient temperatures, a finding which Miyatake et al. (2008) attributed to running away being a more viable energetic option under such conditions compared to when ambient temperatures were low. It is possible that lower temperatures may also, to some extent, reduce the viability of aphids running away or prevent them from reaching their highest possible running speeds (and thereby making TI a more practicable defensive alternative). However, the post-dropping response of aphids to temperature is complicated by metabolic effects and the risk of damage at high levels. Further studies purpose-designed to manipulate temperatures and test greater sample sizes of TI durations across a controlled range of temperatures are needed to explore the effect of temperature on potato aphid behaviour further. Examination of the influence of temperature on TI across other insect groups may also deepen understanding of environmental influences on behaviour, as a study by Ritter et al. (2016) reported species-specific TI frequency gradients associated with rising temperature in *Agriotes* wireworms, some of which followed the opposite direction to the generally assumed trend of cooler temperatures elongating immobility.

The duration of TI exhibited by potato aphids did not appear to be significantly affected by the other variables explored in this experiment. While Wohlers (1981) found that pea aphids exhibited TI for longer when dislodged by tactile stimuli compared to those dropped in response to an approaching coccinellid predator, potato aphids in this current study did not significantly differ in TI durations depending on whether they dropped in response to a foraging predator or the standardised stimulus (contrary to my prediction in Table 6.1). This difference may be due to the fact that in this study the vast majority of drops were triggered by contact with the predator, whereas for Wohlers (1981) the visual and/or vibrational cues of an approaching coccinellid predator were sufficient stimuli to trigger dropping. Perhaps the tactile standardised stimulus in this study was interpreted by aphids as being a similar threat as a predator, as both involved direct contact, and therefore provoked similar TI durations. Similarly, while pea and potato aphids were found to differentiate their use of antipredator defences depending on predator type in Chapters Four and Five, perhaps the lack of difference in potato aphids’ post-drop TI durations between drops induced by the two predator types could also be due to them both involving high-threat contact. The contrast in findings between this current study and that of Wohlers (1981) might also represent a species-specific difference between pea and potato aphids, perhaps resulting from their different life histories and interactions with natural enemies (van Emden and Harrington, 2007, AHDB, 2015). It remains uncertain why different TI durations on the substrate would theoretically be adaptive based on the nature of the stimulus experienced on the plant, given that foliar-foraging predators do not tend to follow dropped aphids to the ground (Wohlers, 1981). Contrary to expectations (see Table 6.1), the TI durations of potato aphids also did not significantly differ depending on the substrate on which they landed. Heat stress poses a threat of desiccation to aphids (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979), and different substrates will heat up and retain moisture to varying extents; I had assumed here that sand may represent a drier, more dangerous substrate. However, as these properties were not measured in the sand and soil substrates used in this current experiment, I cannot be certain that these substrates represented significantly different threats to desiccation. Future studies quantifying the moisture content and temperatures of differing substrates and examining the desiccation risk they pose to dropped aphids are needed in order to test the possible influence of substrate on TI duration further. The maximum height of plants dropped from also did not significantly influence the duration of time TI was exhibited, as I predicted (see Table 6.1). Niku (1975) found that pea aphids exhibit TI for different durations depending on height fallen, in a study that varied drops between 10 and 100 cm. TI duration was negatively correlated with the height fallen (Niku, 1975), possibly resulting from the fact that it is less likely that predators could follow aphids' trajectory or otherwise pursue them if they fall a greater distance, and therefore there is less of a need to remain immobile to reduce predator localization. However, Roitberg et al. (1979) did not find this correlation in their study where pea aphid drops varied in height – by chance – between 10 and 25 cm; the difference in these heights may not have been big enough to translate into significant differences in predators' tracking abilities of dropped prey. In my study, potato aphid drops took place from immature plants whose maximum heights ranged from 9 – 30 cm (closer to the range of Roitberg et al., 1979) and the specific distances fallen by individuals from particular plant parts could not be recorded accurately without disturbing the set-up during assays; for these reasons, it is not surprising that I also found no significant relationship. Finally, I found no significant correlation between the horizontal distance dropped from the original host plant and the time dropped aphids spent in a state of TI. Although I had predicted that greater distances may mean aphids are selected to reduce desiccation risk by rapidly resuming motion towards plants again (see Table 6.1), more relevant variables that would impact how vulnerable aphids assess themselves as being on the substrate include horizontal distance from any plant (rather than their original host alone), light conditions and shading, and the detectability of plants that could be recovered to.

### 6.4.2 Post-drop recovery rates back to original host plants

In line with my prediction (see Table 6.1), aphids showed a pattern of increased recovery rate back to the original host plants with higher temperatures. Combined with the finding above that aphids' TI durations decreased at higher temperatures, this strongly suggests that post-dropping behaviour is adapted to reduce the time spent on the substrate when it is hotter; this makes sense given the risk of desiccation associated with time spent exposed on hot and dry substrate (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979, Dill et al., 1990). Alternatively, or additionally, in both cases higher temperatures also likely increase insect body temperatures, facilitating greater activity and more rapid locomotion. The recovery rate of potato aphids was also affected by stimulus type, with aphids generally recovering back from drops in response to the first standardised stimulus quicker than they recovered after dropping from a predator, as predicted (see Table 6.1). Unlike the differentiation in antipredator defences shown by pea and potato aphids reported in Chapters Four and Five, the recovery rates did not significantly differ between predator types. The slower recovery rate following predator-induced drops may be due to aphids exhibiting a different behavioural pattern once activity was resumed. Wohlers (1981) found that pea aphids dislodged by foraging predators exhibited longer periods of running behaviour before searching for and climbing plant models than those dislodged by touch alone. Potato aphids may similarly switch to searching behaviour more rapidly following tactile stimulus-induced drops compared to predator-induced drops. In a study by Phelan et al. (1976) involving several aphid species (including potato aphids), aphids dislodged by mechanical stimuli oriented rapidly to vertical dowels while those dislodged by exposure to alarm pheromone increased their rate of locomotion, travelled greater distances and changed direction less often. As to the adaptive value of different recovery behaviours, my study was limited by its inability to track specific paths of movement on the substrate and its consideration of only instances where aphids recovered back to their original host plants. Studies such as those by Phelan et al. (1976) and Wohlers (1981) tested aphids in arenas with many plant models and suggest, for example, that running behaviour following predator-induced drops may serve to divert individuals away from original hosts that are dangerously infested with predators. Different triggers of dropping appear to have different consequences for post-dropping locomotion, likely influencing the energetic, time and mortality risk costs of dropping (Losey and Denno, 1998a, 1998b, Nelson, 2007, Agabiti et al., 2016) as well as which plants aphids eventually recover to (Ben-Ari et al., 2015). However, more research is needed into the specific behaviours that potato aphids exhibit during their recovery time in order to fully understand any adaptive value they hold and their fitness consequences in different contexts. It is also interesting to note that while recovery rates back to host plants did seem to vary depending on the nature of the stimulus (suggesting some degree of discrimination between the stimuli) TI duration did not significantly differ following drops due to contact with a predator compared to contact from the standardised stimulus. The significance of the different time aphids spend exhibiting TI and the adaptive value of the behaviour under different circumstances is not well understood (Wohlers, 1981, Bilska et al., 2018), and so future research investigating the relative importance of different stimuli and other selective pressures on variations in both TI and post-TI locomotion will help to unpack this.

The recovery rate of potato aphids that returned to their original host plant after dropping did not appear to be significantly affected by any of the other variables explored in this experiment. In contrast to my prediction (see Table 6.1), the different textures and grain structures of the sand and soil in this experiment did not generate significant differences in recovery rate once aphids had resumed activity; it was predicted that the looser sand substrate would present a greater challenge to aphid recovery than the more compacted composition of the sand–perlite–peat compost mix I considered as soil. Throughout assays I noted that aphids’ ease of mobility did not consistently vary between the two substrate types – both the loosely-packed sand grains and the compacted chunks of soil occasionally caused some individuals temporary issues in locomotion, but mostly aphids appeared to travel with few problems. Substrate may be a more significant issue for post-dropping recovery if younger instars of aphid were tested, as their locomotion is more likely to be impeded by features like stones or grains of soil (Roitberg et al., 1979). Closer study of the effects of the physical qualities of more varying substrates on aphid locomotion might be a useful avenue for future research. Although I did not base my prediction regarding the effect of substrate on recovery rate on its likely temperature or moisture content, it is worth reiterating that these features were not quantified or controlled for in my study. As discussed in Chapter Three, pea aphids have been described as ‘assessing’ the risk of dropping and are less likely to drop when their environment is hot and dry (Dill et al., 1990), suggesting that temperature certainly influences pre-drop behavioural decisions and would likely be an important influence on post-drop assessments of risk too. While ambient temperature appeared to be a key influencer of how vulnerable aphids were to hot and dry conditions and/or how active individuals were in my study, further work is needed to fully understand the contribution of different substrates under different conditions to both of these factors, and the effect of different conditions on the physical characteristics of the substrate itself as the surface over which aphids must travel. For example, following a spell of heavy rain a soil substrate may retain a lot more moisture compared to a sand substrate, potentially enabling dropped aphids to survive for a longer period of time before returning to a host plant; but interactions between substrate moisture content, substrate temperature, and ambient temperature and their influence on aphid behaviours and locomotion abilities need to be explored, including for a wider range of more natural substrate types.

Neither the maximum height of the plants aphids dropped from, nor the horizontal distance aphids landed from their host plant had a significant effect on their recovery rate back to that plant. Theoretically, if insects of a particular species preferred to feed in locations higher up plants, it might be predicted that their recovery rates would be more rapid following drops from taller plants as it would take them longer to re-settle at an appropriate feeding location and they would be selected to minimize unnecessary time spent off-plant. However, in my study the range of maximum heights of the young plants used was not controlled and did not vary greatly, and so I had anticipated that they might not affect recovery rate (Table 6.1). Further, I did not record the within-plant vertical feeding locations aphids dropped from. Concerning horizontal distance dropped, I had predicted that aphids that dropped a greater distance away might experience stronger selection to decrease the length of time they spent exposed to the risks associated with being off-plant (Losey and Denno, 1998a, 1998b, Nelson, 2007, Agabiti et al., 2016), as returning to the plant would unavoidably take longer (Table 6.1). As with the TI duration findings, in hindsight I think that there are alternative variables than horizontal distance dropped that are probably more important to dropped individuals' assessment of risk on the substrate and post-dropping behaviour.

### 6.4.3 Mitigation of dropping/re-clinging/instant recovery

Most drops that preceded re-clinging were triggered by the standardised stimulus rather than predators; this was likely due to the relative infrequency of contact with predators (and subsequent dropping) compared with the standardised stimulus. My consideration of plant position was concerned with the type of plant section an aphid was located on rather than the vertical position up a plant, and – in agreement with my prediction (see Table 6.1) – all but one case of instant recovery followed drops from the leaf undersides. Leaf undersides are more likely to overhang other plant sections than the thinner and more vertical structures of plants, therefore they could be selected by aphids in order to increase their likelihood of instant recovery if dropping becomes necessary; the relative extent of underlying foliage cover was not recorded as part of this experiment, though. The importance of plant position and underlying foliage cover to an aphid's decision to drop and – by extension – its likelihood of re-clinging is discussed in further detail in Chapter Seven, in which I describe fieldwork investigating dropping by wild aphids. Further, or alternatively, the trend of potato aphids to drop from leaf undersides in my study may instead be accounted for by a general tendency of aphids to settle on leaf undersides, perhaps as the most productive feeding sites and/or the sites most sheltered from predator detection and abiotic stress, as the vast majority of aphids settled on leaf undersides (rather than leaf uppersides, stems or petioles) throughout the experiment.

Considering plant factors, the likelihood of aphids re-clinging was not affected by plant age, contrary to my prediction (see Table 6.1). But age was identified as not being a reliable measure of foliage cover or relative plant height across assays. However, maximum height of the immature plants used here (similar to the 'field height' measure used by Meresman et al., 2017) did appear to influence the occurrence of instant recovery in potato aphids as predicted (see Table 6.1), with cases of post-drop re-clinging occurring significantly more commonly on pots with taller plants than shorter plants. This corresponds with previous findings that falling from greater heights increases aphids' abilities to successfully adopt an aerial righting posture (Ribak et al., 2013) and increases the likelihood of aphids re-clinging to underlying plant material (Meresman et al., 2017). Neither the timing of potato aphids' aerial righting nor plants' foliage cover as viewed in vertical direction from above were included in this current study. However, the likelihood of aphids falling from objectively higher positions is greater on taller plants and I would predict that foliage cover would be greater on taller plants, which have presumably grown more – outwards as well as upwards. Plant height and architecture could therefore be important to mitigating the costs of dropping for aphids. In pea aphids, for example, Meresman et al. (2017) have previously demonstrated that re-clinging success is positively dependent on the amount of underlying plant material available for re-clinging and plant height. Particular leaf shapes, plant architecture, and plant growth form also appear to mitigate the costs of dropping for non-aphid insect species; though life stage can have a significant bearing on the necessity for cost-mitigation (and therefore the relative use of dropping across plants with different characteristics) in species whose larvae are flightless but adults are winged (Matsubara and Sugiura, 2018, Matsubara and Sugiura, 2021). Further studies quantifying factors such as foliage cover, plant architecture, and specific pre-dropping positions within individual plants will be valuable to understanding the behavioural ecology of potato aphids and all other species for which post-drop instant recovery remains a relatively new and underappreciated topic. Future work should also consider separating out factors such as plant age and plant height that change concurrently over time, to see how they relate to foliage cover and the likelihood of instant recovery.

### 6.4.4 Conclusions

Through exploring the post-dropping behaviour of potato aphids, an agriculturally-important species that has not received much attention regarding its antipredator defenses, this chapter identified several factors that could have an important influence on tonic immobility, recovery rate, and the likelihood of instant recovery through re-clinging. Future studies with finer-scale measurement and control of key variables such as temperature might reveal even stronger correlations with TI duration and recovery rate. The heights from which aphids dropped could also usefully be investigated more closely in future work. Studies purpose-designed to control the height dropped by aphids (e.g. Niku, 1975) or that have access to sophisticated technology to track distance fallen – rather than using maximum height of plants in a pot as this study did – might find significant correlations with TI duration or recovery rate, or a stronger relationship with the likelihood of re-clinging. Nonetheless, despite the relatively simple measures of temperature and plant height used in this study, significant correlations in the predicted effect directions were found. This indicates that these relationships are highly important, and likely to recur in future studies with more precise measures. I certainly hope that this exploratory study will inspire researchers to design studies to address specific hypotheses about post-dropping behaviour, and to do so for a wider range of species than has been investigated thus far. Such studies utilizing pests of agricultural crops (such as aphids) may be particularly pressing, not only because post-dropping behaviours have the potential to increase dispersal of pests themselves, but they could also increase the dispersal of economically-damaging crop viruses that are transmitted by pests, such as bean yellow mosaic virus (BYMV) (Hodge et al., 2011).

Dropping is an antipredator behaviour that carries nuanced short- and long-term consequences for prey and, as such, dropping is often used in a flexible way in order to minimize the cost; this is discussed in more detail in Chapter Three. The results of this current study, alongside previous work, indicate that post-dropping behaviours can also be complex and utilized in a nuanced way. For example, given that water loss, lost feeding time, and the risk of mortality from ground predators are obvious costs of slow recovery after dropping to the substrate (Losey and Denno, 1998a, 1998b, Nelson, 2007, Agabiti et al., 2016), it might be assumed that individuals would travel back to a plant as quickly as possible. However, the process of falling some distance could well attract nearby mobile visual predators and therefore a short display of TI could be important to reduce localization of prey (Miyatake et al., 2009). Such TI itself, though, increases time off-plant and will carry a greater risk of desiccation when the environment is hotter and drier; though the humidity and moisture content of the environment and substrate were not explored in this current study, they should be considered in future, purpose-designed work. Immediate return to plants, therefore, might not always be the most adaptive option, but aphids must trade-off conflicting pressures associated with post-drop recovery. The easiest way for aphids to mitigate any costs of dropping (while still evading an approaching threat on the plant) is to re-cling to underlying plant material, but this is only possible under certain conditions. While the fitness of aphids in the wild will be determined primarily through repeated rather than one-off predator encounters, developing understanding of the behavioural variation following dropping defense under different conditions will allow the prediction of longer-term and larger-scale consequences for both prey and predator populations and their broader ecologies.

# Chapter 7: Ladybird searching behaviour and antipredator dropping by wild aphids: a field study

The searching behaviour of predators through their environments influences their predation efficiency and success, but the antipredator defences of their prey will also have a significant bearing on the outcome of any predator-prey interactions. Both 'sides' to such interactions have separately been the focus of many studies utilising the ladybird-aphid predator-prey system, but research rarely reports behaviours recorded under natural conditions or considers both sides in concert. The exploratory field study reported here aimed to generate hypotheses and ideas for future studies based on observations of ladybird searching behaviour and dropping by wild aphids in a natural context. Experimental trials constituted observations of the movements, frequency of contact with prey, and predation success of ladybirds introduced onto wild plants with aphid prey present, and the frequency of aphid antipredator dropping in relation to: broad aphid age, contact with ladybirds, the plant structure on which dropping took place, and weather conditions. Overall, ladybirds did not appear to search in a way that would efficiently lead them to prey when searching wild plants. Further, although ladybirds commonly made contact with prey, encounters relatively rarely ended in successful prey capture. Considering the prey, aphids only rarely exhibited antipredator dropping behaviour as an escape from their plant section, making it difficult to draw strong conclusions in relation to the variables considered. Adult aphids appeared more likely to drop than nymphs, and dropping likelihood may have depended to some extent on whether aphids occurred on plants with dense understructure; but further work is needed to explore these suggestions. More generally, the findings of this study raise many questions for future research to explore in more purpose-designed studies, and avenues of inquiry that might be especially valuable are highlighted.

## 7.1 Introduction

How predators search for prey in their environments and how prey react to the presence of predators hold significant consequences for the fitness of predators and their prey at both individual and population scales. Aphidophagous ladybirds (Coleoptera: Coccinellidae) are key predators of aphids, which themselves are economically-important phloem-feeding pests of agricultural crops across the globe (van Emden and Harrington, 2007, Dedryver et al., 2010), as discussed in Chapter Two and the Appendix. Ladybirds are considered effective biological control agents, due to their voracious consumption of a wide range of prey, including aphids (Hodek and Honek, 1996, Obrycki and Kring, 1998, Dixon, 2000). Aphids, however, are not defenceless; they have been found to exhibit a range of antipredator defensive behaviours against their natural enemies, ranging from kicking or walking away (Dixon, 1958, Clegg and Barlow, 1982, Brodsky and Barlow, 1986, Dion et al., 2011), to dropping away from their current plant section (Roitberg and Myers, 1978, Agabiti et al., 2016, Harrison and Preisser, 2016). Indeed, Chapters Four, Five, and Six described experiments I conducted to explore the use of these antipredator behaviours – particularly dropping – by aphids under a range of different conditions. However, many studies exploring coccinellid foraging efficiency do not consider the use of particular ladybird searching or aphid antipredator behaviours; instead, ladybirds are left to search infested plants for a set length of time before the number of surviving prey are counted (Carter et al., 1984, Francke et al., 2008, Cabral et al., 2009, Cabral et al., 2011, Omkar and Ahmad, 2011, Reynolds and Cuddington, 2012, Al-Deghairi et al., 2014). Some studies have examined the short-term sequences of coccinellid behaviour when searching environments and interacting with aphids, but most have taken place under laboratory conditions (Kauffman and Laroche, 1994, Vohland, 1996, Clark and Messina, 1998, Elliott et al., 2011a, Elliott et al., 2011b), with a few exceptions involving potted plants outdoors (e.g. Grevstad and Klepetka, 1992, Minoretti and Weisser, 2000). Exploratory studies on wild plants may provide useful insights as to ladybird searching behaviour and aphid antipredator defence in more naturalistic contexts, particularly as significant differences have been reported between laboratory-observed and field-observed ladybird-aphid predation relationships (Frazer and Gilbert, 1976, Latham and Mills, 2009).

In terms of what is currently known about ladybird searching behaviour, it is thought that ladybirds forage both extensively and intensively (Ferran and Dixon, 1993). Extensive search occurs when ladybirds move between aggregates of prey within patches (defined as an area where the probability of encountering prey is relatively high compared to surrounding areas), but switches over to intensive (or area-restricted) search when ladybirds move between prey individuals within aggregates of aphids (Hassell and Southwood, 1978, Ferran and Dixon, 1993). Extensive search is characterised by relatively fast, linear movement, while intensive search involves slower movement with an increased frequency of turns and pauses, the adaptive function of which is considered to be keeping the predator in the vicinity of the prey aggregate (Dixon, 1959, Ferran and Dixon, 1993). Contacting or consuming an aphid appears to be a key cue that elicits the change from extensive to intensive search by aphidophagous ladybirds (Nakamuta, 1985, Ferran and Dixon, 1993). If another prey individual is not found within a short period of intensive search, ladybirds switch back to extensive search (Marks, 1977, Carter and Dixon, 1984). While the switching between search behaviours was first reported for coccinellid larvae (Banks, 1957, Dixon, 1959), it is thought that extensive and intensive search are characteristic of ladybird adults too, as well as other predators and parasitoids (Nakamuta, 1985).

Early studies based on larvae concluded that ladybirds do not appear to perceive their prey before making contact (e.g. Banks, 1957) and observations of apparently aimless walking by adults led to them being described 'blundering idiots', as pointed out by Hodek and Evans (2012) (Pervez and Yadav, 2018). But later works indicate that coccinellid foraging is not undirected. For example, ladybird adults have been demonstrated to visually perceive prey at close proximity (Nakamuta, 1984, Lambin et al., 1996, Lim and Ben-Yakir, 2020); and, for some species, lighting and background colour can influence predation efficiency (Harmon et al., 1998). Olfactory cues relating to aphid presence, including aphids themselves and aphid-damaged plants also seem to guide ladybird movements (Obata, 1986, Ninkovic et al., 2001, Pettersson et al., 2008, Pervez and Yadav, 2018), with a recent study finding that *Coccinella septempunctata* adults utilise olfactory cues to locate aphid-infested plants when foraging nocturnally (Norkute et al., 2020). Interestingly, ladybirds have even been found to detect and respond to the quality of their prey's resource, selecting high-quality patches regardless of prey presence (Williams and Flaxman, 2012).

Considering how ladybirds explore plant structures more broadly, rather than when travelling within a patch, aphidophagous coccinellids have been found to exhibit positive phototaxis and negative geotaxis, both of which help lead them to the terminal parts of plants where aphids are most likely to be found (Dixon, 1959, Frazer and McGregor, 1994). In Chapters Eight and Nine, I describe experiments I conducted to further investigate how ladybirds forage on plant structures ahead of encountering any prey, focussing on the potential use and adaptive benefits of turning biases and preferences. On plant leaves, physical cues also seem important to plant exploration, with ladybird foraging focussed primarily along leaf edges and dominant veins rather than leaf laminae (Dixon, 1959, Marks, 1977, Ferran and Dixon, 1993); though less is known about the differential predation risk aphids face at these different sites (Keiser et al., 2013).

The purpose of the exploratory study reported here was to generate hypotheses and ideas for future studies based on observations of ladybird searching behaviour and dropping by wild aphids in a natural context. As explained in Chapter One, this study essentially serves as a bridge between the two halves of this thesis, bringing together the aphid antipredator dropping ‘side’ (covered in Chapters Three, Four, Five, and Six) and the ladybird searching ‘side’ (covered in Chapters Eight and Nine) in its consideration of predator-prey behavioural interactions on wild plants. For the ladybird searching component, the aim was to explore a range of broad questions:

i. Do ladybirds choose to turn onto plant sections with aphids present? This may indicate whether ladybirds are able to detect and preferentially select plant sections with aphid prey on from a distance.
ii. Do ladybirds initially head in the direction of aphids once on a plant section with aphids present? This may indicate whether ladybirds use cues (potentially visual or olfactory) to target aphids when they first move onto a plant section where aphids are present.
iii. How commonly do ladybirds make contact with, and successfully capture, aphids? Given the importance of contact as a cue to elicit the switchover from extensive to intensive search (Nakamuta, 1985), the relationship between contact and successful predation is worthy of further exploration.
iv. Is successful predation more likely to occur early on in a ladybird’s search of a plant section, and is success likelihood affected by initial trajectory? This may indicate how important ladybirds’ abilities to initially detect, and potentially target, prey are to their success as predators.
v. Does total time spent on a plant section correlate with instances of contact? This may give a general indication of how thorough and effective ladybirds are at searching plant sections and/or pursuing prey up until a point where they succeed in capturing an aphid.
vi. Does experience of a foraging scenario increase ladybirds’ chances of successfully capturing prey? Given that individual ladybirds in this study could be tested up to a maximum of three times, it was worth considering whether they showed any learning or improved predation abilities with experience.

For the aphid response component, dropping behaviour was the focus due to its relatively underappreciated status as an antipredator defence (as discussed in Chapter Three). This study was interested in exploring the following questions:

i. How often does antipredator dropping occur and does this relate to the plant structure aphids start on, and/or the presence of underlying vegetation? In Chapter Six, I found that potato aphids primarily dropped from lower leaf positions and that the likelihood of potato aphids re-clinging to plant material after dropping may be greater on taller plants. This could be due to taller plants presenting aphids with greater underlying foliage cover (Meresman et al., 2017), a trait that might make dropping more practicable given that this enables dropped aphids to avoid falling to underlying substrate and experiencing associated costs (discussed in Chapter Three). Plant structure and underlying vegetation, and how they relate to re-clinging likelihood, may therefore influence how likely aphids are to drop.
ii. Do nymphs or adults drop more, and does dropping occur more pre- or post-contact? As discussed in Chapter Three, insects of various taxa often exhibit ontogenetic differences in the use of dropping defence. Whether nymphal stages are less likely to drop than adults due to their greater vulnerability off-plant, or more likely to drop due to a reduced ability to kick, run away, or otherwise defend themselves has been little explored in wild aphids. In Chapter Five, I found that contact with a predator was a key trigger for dropping in both pea and potato aphids, but how far this applies to other species of aphid under wild conditions is not known.
iii. Is dropping any more or less likely under different weather conditions? While aphids are thought to drop less under hot and dry conditions (discussed in Chapter Three), most experiments into the influence of environmental conditions have not taken place under natural conditions or considered weather conditions such as wind, cloud cover, or rain.

Because the study was exploratory in nature, convenience sampling was employed in order to collect data on a range of aphid species on a range of plant species.

## 7.2 Materials and methods

### 7.2.1 Ladybird predators

45 adult two-spot ladybirds (*Adalia bipunctata*) were supplied by Green Gardener (Rendlesham, Suffolk). Ladybirds were maintained in ventilated plastic containers of various size in groups of no more than 10 individuals, to reduce the chances of cross-infection. Containers were supplied daily with a fresh piece of damp kitchen towel and pieces of grape as a food supplement. Individuals could be used in trials a maximum of three times, but never completed more than one trial in a single day. Fresh individuals, individuals trialled once and individuals trialled twice were stored in separate containers, such that ladybirds were not individually kept track of, but for each individual it was known how many trials it had undergone. No ladybird was kept for more than four days, and all were released as soon as no longer required for the study.

### 7.2.2 Experimental protocol

Fieldwork took place over ten days at the start of August 2020 in Kelvingrove Park, along Kelvin walkway, and along the last 2km of the River Kelvin in Glasgow, UK (LAT: 55.87, LONG: -4.28). For each trial a plant section with a wingless individual aphid, or a group of wingless aphids present was located – referred to as ‘the plant section of interest’. The plant genus was identified using the PlantNet app (Pl@ntNet, 2020), and the number of adults and nymphs present was estimated based on the size of each individual. As a measure of how likely re-clinging might be for aphids that dropped, the quantity of underlying plant matter below the plant section of interest but higher than the ground (i.e. not including grass if this was the underlying substrate) was scored as absent, sparse, or dense. It was recorded whether rain was absent, light, persistent or heavy, and how many eighths of the sky were cloudy. Observations from the nearest Met-Office station were checked after trials to determine the local temperature and average wind speed at the hour nearest to when each trial took place.

At the beginning of each trial, an audio recording was started on a mobile phone. In most cases, the plant section of interest was the only section in the immediately-surrounding plant structure where aphid prey were present (that is, several other sections would have to be traversed and multiple turning decisions made from the section of interest in order to locate an alternative prey patch). In these cases, a ladybird was introduced to the plant, using a paintbrush, at the base of the plant section positioned one turning decision away from the plant section of interest itself or to the structure leading exclusively to the plant section of interest, whichever of these enabled the recording of a turning decision towards or away from the section with aphid prey present. For example, if the section of interest was a lower leaf, the ladybird would be introduced to the stem below where the petiole heading exclusively towards the leaf branched off (see point A in Figure 7.1), or the petiole before the petiolule in the case of compound leaves. Whether the ladybird moved onto the plant section of interest (or the structure leading exclusively to the plant section of interest, see point B in Figure 7.1) or onto the alternative, ‘no aphids’ option at the turning point which would not lead them to aphid prey (see point C in Figure 7.1) was dictated to the recording. If a ladybird turned around before making a turning decision, it was repositioned at the base of the section before the turning decision (see point A in Figure 7.1). If a ladybird chose the alternative ‘no aphids’ option, it was positioned at the base of the plant section of interest (see point D in Figure 7.1). In the relatively few cases where there were aphid prey present along the alternative option as well as the plant section of interest, this section of trials was skipped and the ladybird was introduced directly to the base of the plant section of interest (see point D in Figure 7.1).

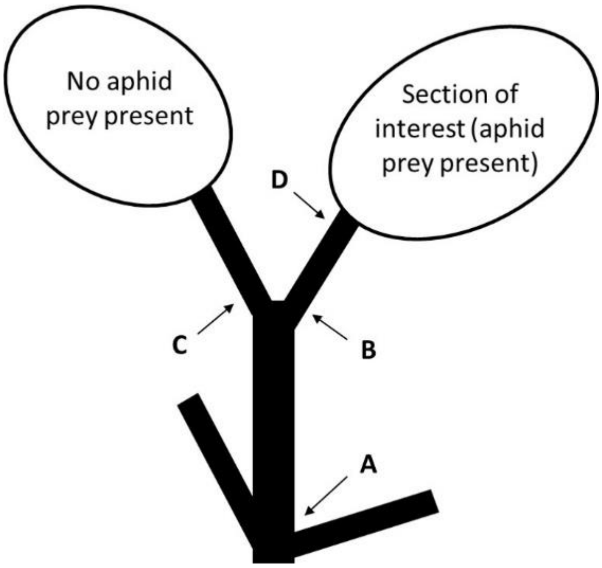


**Figure 7. 1:** Diagram highlighting key points (A-D) where actions were taken or ladybirds could make turning choices during the early stages of trials, prior to ladybirds exploring the plant section of interest.

Once at the base of the plant section of interest, either following a choice towards that section or from being introduced directly, the time was dictated to the recording. Where the plant section of interest was the upper or lower side of a leaf (which was most trials), the leaf overall was considered as being part of the plant section of interest for the remaining protocol, given that they are structural dead-ends on plants and ladybirds might explore both upper and lower sides before heading back down a petiole or petiolule. Whether the ladybird headed towards or away from aphid prey on that plant section in its first few steps was recorded. If aphid prey were located somewhere within an estimated 45-degree-span of the ladybird's initial trajectory this was classed as ladybirds heading 'towards' the direction of prey, but otherwise ladybirds were considered to be heading 'away' from prey. If a ladybird initially moved onto an upper leaf when aphids were located on the lower leaf (or vice versa), the time the ladybird moved onto the leaf overall was still considered the time that the ladybird started searching (as the leaf overall was considered as the same gross section), but its initial 'heading' towards or away from prey was only recorded when it moved onto the surface with aphid prey on. Ladybirds were given a maximum of three minutes to explore the plant section of interest. Activity dictated to the recording during this time comprised instances (for both adult and nymph aphids) where:

i. individual aphids dropped pre-contact with predator
ii. contact was made between an aphid and the predator and the aphid dropped immediately
iii. contact was made between an aphid and a predator and the aphid did not drop immediately but did drop before any further contact was made
iv. an aphid dropped after being contacted by the predator multiple times
v. contact was made once between an aphid and the predator but the aphid did not drop
vi. contact was made between an aphid and the predator multiple times, but the aphid still did not drop
vii. a ladybird successfully captured an aphid (which also corresponded with one of the cases above where contact was made but the aphid did not drop).

The trial and audio recording were ended after either:

i. the ladybird grabbed and started consuming an aphid
ii. the ladybird left the plant section of interest
iii. all of the aphid prey on the plant section left
iv. the three minutes elapsed.

If the ladybird left the plant section of interest within a minute without encountering any aphids, the ladybird was repositioned at the base of the plant section of interest and the part of the trial where the ladybird was on the relevant section was considered as re-started. If a ladybird left the plant section of interest within a minute without encountering any aphids during this re-started trial, its longest attempted search was classed as the final trial. After a trial ended, the length of time the ladybird searched the plant section of interest was calculated from the recording, and the number of aphids that remained on the plant at the end of the trial was counted (regardless of whether any contact was made with them or whether they walked away to different plant sections during the trial – so long as they did not drop). Seventy trials were completed in total. All analyses were conducted using R version 3.6.3 (R Core Team, 2017).

## 7.3 Results

### 7.3.1 Ladybird searching

#### *7.3.1.1 Do ladybirds choose to turn onto plant sections with aphids present?*

There were 18 trials where ladybirds were introduced directly to the plant section of interest because the alternative route also led to a plant section with at least one aphid on. In all other trials, ladybirds were introduced to the base of the plant section below the turning point where they could then choose between moving onto the plant section of interest (with at least one aphid present) or to a plant section without any aphids on. In 36 trials ladybirds chose the ‘non-aphid’ option when encountering this choice, while in only 16 trials ladybirds chose the option leading to at least one aphid ($\chi^2_1 = 7.7$, $P = 0.006$). Often the choice for the ‘non-aphid’ option correlated with a choice for remaining on a more structurally substantial plant part, such as the stem or a main branch of the plant. Correspondingly, a choice of moving onto a section with aphid prey would commonly involve turning off from the current heading, and down a thinner plant structure, such as a petiole (or petiolule in the case of compound leaves). The likelihood of ladybirds choosing the plant section of interest over the ‘non-aphid’ option may not, therefore, be a simple 50:50 chance at two-option decision points on plants.

#### *7.3.1.2 Do ladybirds initially head in the direction of aphids once on a plant section where they are present?*

Once on the section of the plant with aphid prey present, in their first few steps ladybirds headed towards at least one aphid (that is, an aphid was present in a 45 degree-span from the ladybird’s trajectory) in 23 trials, but headed away from where aphids were located in 47 trials ($\chi^2_1 = 8.2$, $P = 0.004$). For the 23 trials where ladybirds appeared to head towards aphids, often they would soon turn away from the prey again, so their original heading may have been undirected and unintentional. The more aphids there were on a plant section (and the smaller the plant section), the higher the chance a ladybird's first steps would be in the direction of at least one of the aphids, and the higher the chance it might contact the aphids. In 16 of the trials where ladybirds initially headed in the direction of aphid prey, at least one aphid was contacted by the predator as it followed along its initial trajectory on the plant section, but in only five of these cases an aphid was immediately grabbed and consumed during this initial encounter. In two further cases, aphid prey that a ladybird initially headed towards was grabbed and consumed at later points in the trials. However, whether the initial heading had any bearing on these captures is unclear. In three other trials, ladybirds that initially headed towards aphid prey did successfully capture an aphid, but not one that they initially turned in the direction of.

Of the five instances where ladybirds headed towards and immediately grabbed prey, two of these occurred on petioles or petiolules, rather than leaves, sections of plants where a ladybird was far more likely to encounter prey (as there was far smaller surface area). Another case was on a notably small birch leaf with multiple aphids present. A further case involved four nymphs that were clumped at the very base of a rose leaf. And in the final case, the adult aphid that was grabbed on a bramble leaf was located towards the tip of the leaf but in the middle of the leaf's central vein. Ladybirds commonly searched leaves primarily by following round the edges of the leaf, on the upper and lower surface, and by moving along the central veins of the leaves. Their initial heading was typically either to the edge of the leaf on one side of the petiole, if they were edge-following, or straight ahead down the leaf's central vein. It therefore is not surprising that the aphids that were captured from ladybirds following their initial trajectories were located on sections with small surface areas (where the ladybird had little movement options), or close to the base of leaves, or along the central vein.

#### *7.3.1.3 How commonly do ladybirds make contact with, and successfully capture, aphids?*

In 50 trials (71%) ladybirds made contact with at least one aphid on the plant section of interest, and in 21 of these at least one aphid was contacted by the ladybird multiple times during the trial. Despite this, only 13 trials resulted in ladybirds successfully capturing prey. The four adult aphids that were grabbed and consumed during trials were captured during the ladybird's first and only contact with an aphid on the plant section of interest. The nine nymphs that were grabbed and consumed during trials were rarely the only individuals to have been contacted by the ladybird during their trial. Ladybirds made contact with 40 individual adult aphids overall (114 adults were present across trials, 35% were contacted) and 40 individual nymphs (172 were present across all trials, 23% were contacted).

#### *7.3.1.4 Is successful predation more likely to occur early on in a ladybird's search of a plant section, and is success likelihood affected by initial trajectory?*

It appears that, regardless of whether ladybirds' initial heading was 'towards' or 'away' from the direction of any aphid prey, most successful captures of aphids by ladybirds occurred earlier on in trials (Figure 7.2). However, the sample sizes of successful captures are small and the difference does appear to be less significant when the initial direction was 'away' from any aphids (Figure 7.2); the 'towards' cases of successful capture include the five that occurred almost immediately from the ladybird's initial trajectory.

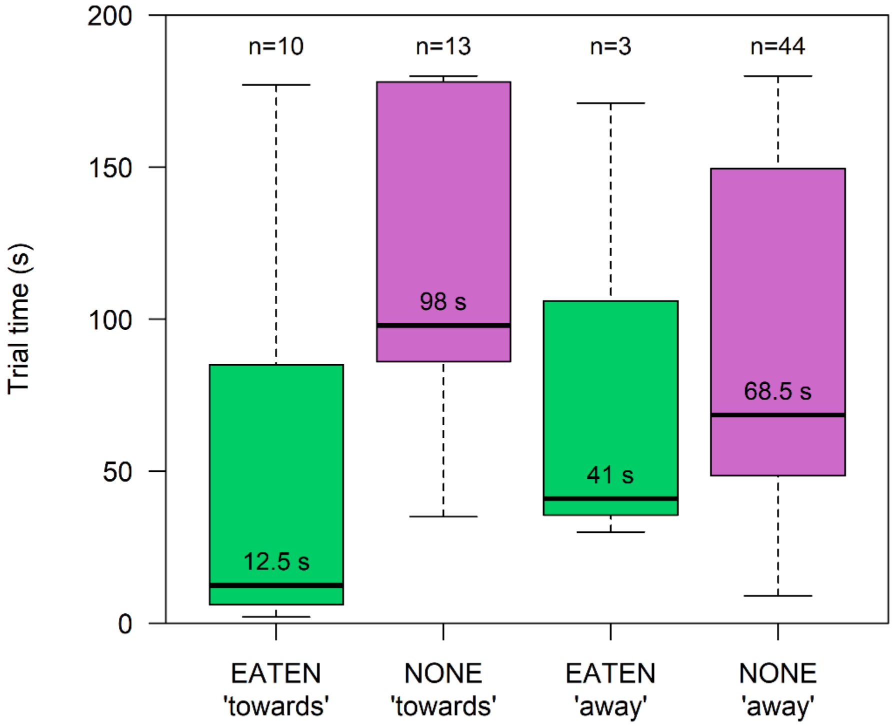


**Figure 7. 2:** The different lengths of trials (in seconds) where ladybirds successfully predated an aphid (EATEN) and failed to capture an aphid (NONE), where their initial heading on the plant section of interest was 'towards' or 'away' from any aphid prey present on the section. The number of trials during which the different scenarios occurred is stated above the boxplots, and the median trial time is stated within the boxes.

### *7.3.1.5 Does total time spent on the plant section correlate with instances of contact?*

Whether trials ran to the full three minutes, or were cut short due to the ladybird leaving the plant section or successfully grabbing an aphid, the raw frequency of aphids that were contacted slightly increased the longer trials lasted (Figure 7.3a). This slight positive correlation appears to apply both to trials where no individual was contacted more than once, and where at least one individual was contacted multiple times (Figure 7.3a). However, Kendall’s rank correlation tau did not find the relationship to be significant for the trials where no individual was contacted more than once (z = 1.57, P = 0.116) or the trials where at least one individual was contacted multiple times (z = 0.93, P = 0.352).

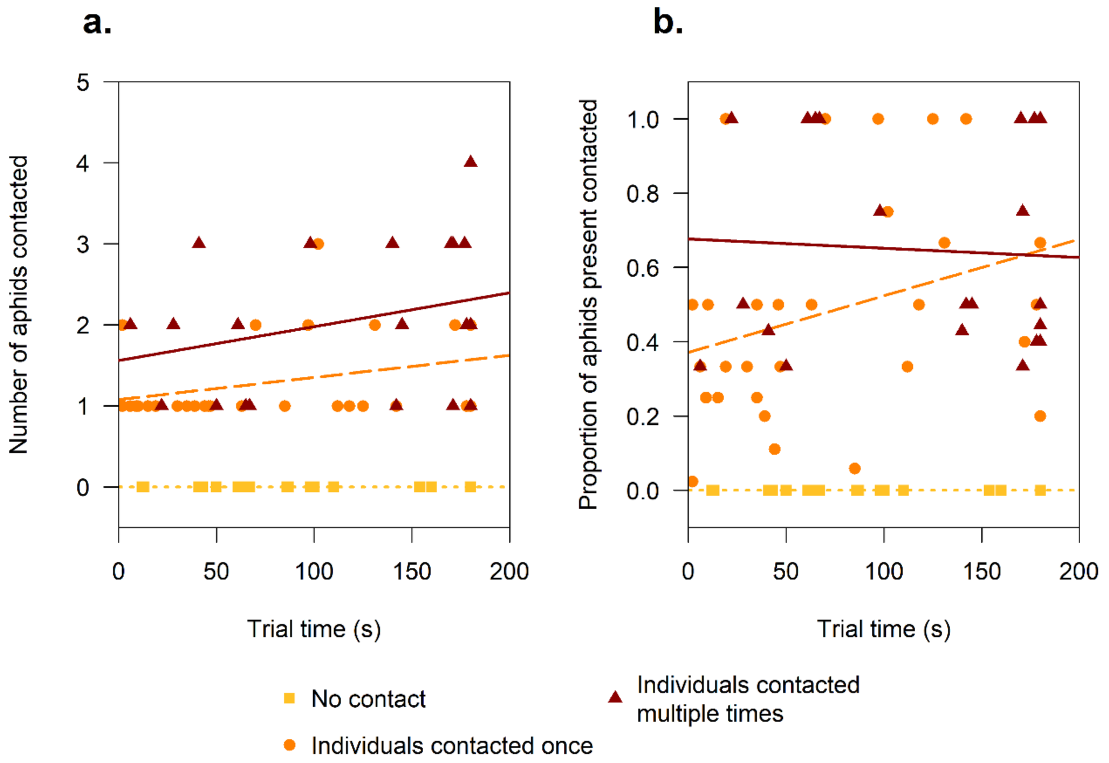


**Figure 7. 3:** Aphids contacted by ladybirds over trials of different length, where **a)** shows the raw frequency contacted and **b)** shows the proportion of aphids present on the plant section of interest that were contacted. Yellow squares denote trials where ladybirds never contacted any aphids (n = 20, linear model of trend as dotted yellow line), orange circles indicate trials where ladybirds contacted at least one aphid individual once but never contacted a single individual multiple times (n = 29, linear model of trend as dashed orange line), and dark red triangles denote trials where ladybirds contacted at least one individual aphid multiple times (n = 21, linear model of trend as solid dark red line).

Considering the proportion of aphids that were contacted relative to the total number present on each given plant section, only for trials where no individual was contacted more than once was there an increase with trial time (Figure 7.3b). Though a Pearson's product-moment correlation did not find this relationship to be statistically significant (t = 1.68, df = 27, P = 0.105). For trials where at least one individual aphid was contacted multiple times, the proportion of aphids contacted slightly decreased with the length of trials (Figure 7.3b), though again this correlation was not found to be statistically significant (t = -0.25, df = 19, P = 0.804).

#### *7.3.1.6 Does experience of a foraging scenario increase ladybirds' chances of successfully capturing prey?*

The aphids that were consumed during trials were captured by 'fresh' ladybirds on nine occasions (69%), 'second use' ladybirds on three occasions (23%) and a 'third use' ladybird on one occasion (8%). The distribution of these frequencies did not differ significantly from the distribution of usage of ladybirds across all trials: 45 fresh, 19 second use, six third use ($\chi^2_2$ = 0.14, P = 0.93). However, the number of eaten aphids was so few that the assumptions of the chi-square test were violated. A Fisher's exact test comparing the relative proportions of these counts, though, also found no significant difference (P = 1). Ladybirds did not seem to improve their predation likelihood with increased experience of the trial format.

### 7.3.2 Aphid dropping

#### *7.3.2.1 How often does antipredator dropping occur, and does this relate to the plant structure aphids start on, and/or the presence of underlying vegetation?*

Only seven aphids dropped to escape from their current plant section across all 70 trials. Dropping occurred from:

- a nettle lower leaf with dense vegetation understructure
- two nettle lower leaves with sparse understructure
- one nettle's flowers with dense understructure
- a small birch lower leaf with sparse understructure
- a birch lower leaf with no understructure
- an elm lower leaf with no understructure.

Four of the seven (57%) drops occurred on nettle plants, although trials involved nettle plants only 24% of the time. Perhaps this is due in part to the fact that a relatively high proportion of trials with nettle plants had dense plant matter underlying the plant section of interest (Figure 7.4) and, from observation, this dense understructure consisted mostly of other nettle plants (for many of the trials with other plant genera, the understructure recorded consisted of plants of genera different to that of the host plant). In the trial where an aphid dropped from the nettle lower leaf with dense understructure below, this was a nymph that dropped ahead of contact with the ladybird (and this was the only case of a nymph dropping) but was seen to re-cling onto a bundle of nettle flowers from the same plant close below the original plant section. However, with so few drops recorded it is not possible to confidently say whether the presence or absence of understructure influenced dropping likelihood.

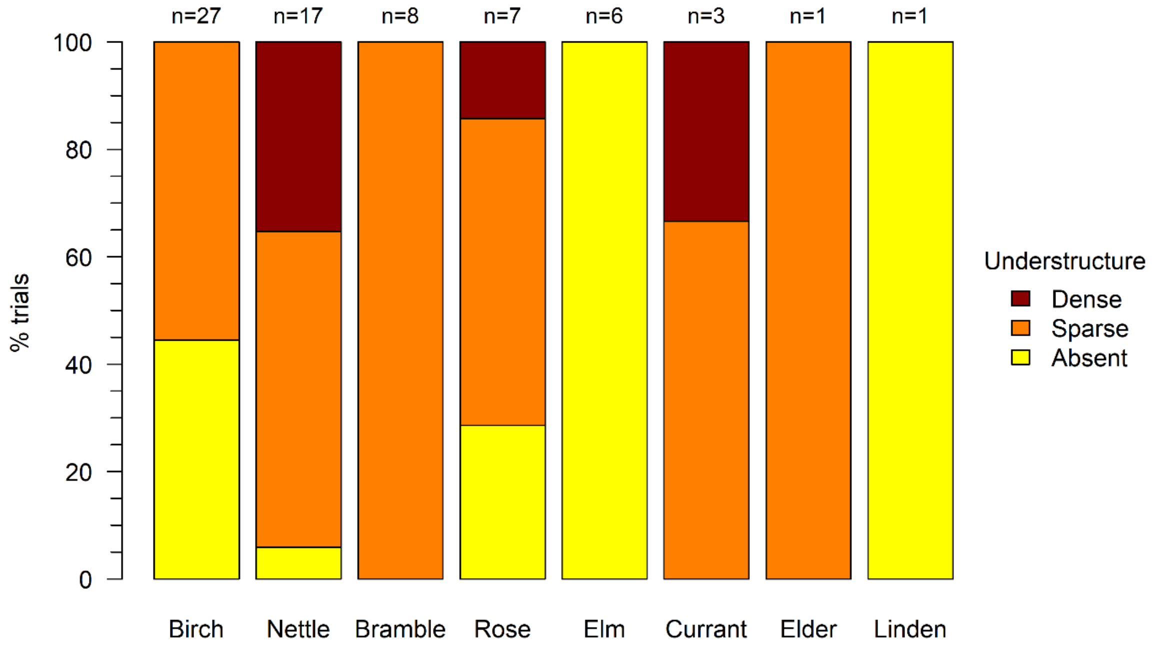


**Figure 7. 4:** The relative proportions of trials on different plant genera where the vegetation understructure was scored as absent (yellow), sparse (orange), or dense (dark red). Genera are ordered by descending number of trials performed on them, with the number of trials on each genera stated above the bars.

#### *7.3.2.2 Do nymphs or adults drop more, and does dropping occur more pre- or post-contact?*

Six of the recorded drops were by adult aphids and only one recorded drop was by a nymph. As mentioned above, the trial where the nymph dropped ahead of contact with a ladybird occurred on a nettle lower leaf with dense understructure below, and the nymph was seen to re-cling onto a bundle of nettle flowers from the same plant close below. Two adult aphids dropped ahead of contact with the ladybird, both from birch lower leaves, but one of these dropped after being bumped into by another adult that was quickly running away from the ladybird after having been contacted by it. Another two of the adults dropped immediately after having been contacted by the ladybird, from right beside the ladybird. One of the adults dropped after a slight delay, having ran away from the ladybird a short distance after contact was made. The final dropped adult was contacted twice by the ladybird before dropping.

More drops occurred post-contact with a ladybird (57%) than pre-contact, and most drops were by adult aphids (86%) rather than nymphs, although a higher proportion of adult aphids present across all trials (35%) were contacted by ladybirds compared to the proportion of nymphs present across all trials that were contacted (23%).

#### *7.3.2.3 Is dropping any more or less likely under different weather conditions?*

Weather conditions did not vary strongly over the ten days in August during which trials took place. Firstly, rain was absent for 68 of the 70 trials and only light in the remaining two. A Fisher’s exact test therefore found no significant difference in the proportion of counts of trials with dropping by aphids under different rain conditions (absent: 7, light: 0) compared to the proportion of counts of different rain conditions across all trials (P = 1). Secondly, skies were mostly filled with clouds across all trials (see Table 7.1). A Fisher’s exact test found no significant difference in the proportion of counts of trials with dropping by aphids under different cloud conditions (see Table 7.1) compared to the proportion of counts of different cloud conditions across all trials (P = 0.40). Considering temperature, a Welch two-sample t-test found no significant difference (t = 0.32, df = 8.73, P = 0.76) between the mean temperature during trials in which an aphid dropped (min: 16°C, median: 19°C, mean: 18.43°C, max: 20°C) and the mean temperature during all trials (min: 14°C, median: 19°C, mean: 18.24°C, max: 21°C). A Wilcox rank sum test with continuity correction also found no significant difference between the median temperatures (W = 233, P = 0.83). Finally, looking at the wind, a Welch two-sample t-test found a significant difference (t = -2.7, df = 10.36, P = 0.02) between the mean wind speed during trials in which an aphid dropped (min: 3.0 mph, median: 7.0 mph, mean: 6.6 mph, max: 8.0 mph) and the mean wind speed during all trials (min: 3.0 mph, median: 8.0 mph, mean: 8.8 mph, max: 16 mph). However, a Wilcox rank sum test with continuity correction found no significant difference between the median wind speeds (W = 160, P = 0.13).

**Table 7. 1:** Frequency of all trials and trials where dropping by aphids occurred under different cloud conditions.

| **Eighths of sky filled with cloud** | **No. trials** | **No. trials where dropping occurred** |
|---:|---:|---:|
| 0 | 9 | 1 |
| 1 | 5 | 0 |
| 2 | 5 | 1 |
| 3 | 2 | 0 |
| 4 | 3 | 1 |
| 5 | 4 | 0 |
| 6 | 5 | 2 |
| 7 | 4 | 0 |
| 8 | 33 | 2 |
| **Total:** | 70 | 7 |

## 7.4 Discussion

One of the key findings of this study was that ladybirds did not commonly appear to make decisions that would successfully lead them quickly to prey when searching wild plants. When given a choice of plant sections to explore, ladybirds more frequently chose to move onto the plant section that did not have any aphid prey present rather than move onto the section with prey present. This indicated that ladybirds do not seem able to sense prey presence (or plant traits that correlate with prey presence) on nearby but unexplored plant sections. In many cases, the route-choice options leading to aphids would require ladybirds to turn off from a main branch or stem and head down a petiole or petiolule. A preference for thicker plant sections, or sections that do not deviate as much from their current heading may explain this trend. Indeed, in Chapter Nine I describe a study that finds evidence for two-spot ladybirds exhibiting preferences for thicker over thinner, straighter over more deviating, and higher over lower headings at bifurcations on branched structures. It is possible that such a preference may be adaptive if it ultimately helps ladybirds explore plants or reach their prey more efficiently. Previous studies have reported aphidophagous coccinellids as being positively phototaxic and negatively geotaxic, traits that help lead them to aphids on the terminal parts of plants (Dixon, 1959, Frazer and McGregor, 1994). In this current study, though, gravitational direction and relative light intensity were not recorded, and the preference for thicker and straighter sections might not always have correlated with those previously-reported tendencies (and the tendencies found in Chapter Nine). The findings of this study could also simply result from sensory limitations of ladybirds, if they are unaware of alternative branching options as they move along some plant structures. Certainly some ladybirds contacted both turning options before making a turning decision one way or another, but there were other cases where the ladybird did not seem to notice the alternative route available. Given that ladybirds have been reported to exhibit visual discrimination and recognition abilities (Nakamuta, 1984, Lambin et al., 1996, Harmon et al., 1998, Lim and Ben-Yakir, 2020) and detect odours from aphid-damaged plant parts (Obata, 1986, Ninkovic et al., 2001, Pervez and Yadav, 2018, Norkute et al., 2020), a more controlled, quantitative study into the choices of ladybirds at turning junctures with varying prey presence would be useful. It is possible that ladybirds in this study made undirected turning choices due to their very recent introduction to the plant structure; perhaps they did not have sufficient time to orient to their surroundings and pick up on the long-distance cues of herbivore presence. Efforts to test wild rather than commercially-cultured ladybirds are also required in order to check whether the apparent inefficiency of ladybird search observed accurately reflects natural searching behaviours, as long-term culturing of insect species can sometimes result in domestication and behavioural changes (Thieme and Dixon, 2015).

The trials in this study were most commonly based on the leaves of plants, and once ladybirds had moved onto these plant sections with aphid prey present they mostly initially headed in a direction that suggested that they could not immediately detect the aphid prey present. More generally, ladybirds often spent most of their searching time tracing round the edges of leaves or moving along the central vein of leaves, as reported previously (Dixon, 1959, Marks, 1977, Ferran and Dixon, 1993), though this was not quantified in my study. Searching along leaf veins is likely adaptive as this can often be where aphid density is highest (Dixon, 1959, Wratten, 1973) but, where leaves lack prominent veins, coccinellids' tendency to follow leaf edges or petioles might mean their search does not stay focussed in areas of highest aphid density (Carter et al., 1984, Ferran and Dixon, 1993). Future studies might consider tracking the time ladybird predators spend searching different areas of plant sections (such as leaves) and the amount of time aphids tend to occupy those different areas of plants, breaking down sections such as lower leaves into smaller components such as dominant veins, leaf edges, and leaf laminae; potentially more detailed and sub-section-specific versions of the methods Salyk and Sullivan (1982) or Clark and Messina (1998) used to quantify distributions of aphid prey would be useful here. Keiser et al. (2013) assessed the life history and predation risk consequences of within-leaf feeding site choices made by pea aphids (*Acyrthosiphon pisum*), and found that aphids feeding closest to the leaf petiole were at the greatest risk of predation by a ladybird. They propose that the tendency of ladybirds to search along leaf edges and veins occurs only after prey are not discovered at proximal leaf sites. From this, aphids (particularly the vulnerable earlier instars) might be predicted to preferentially feed nearer the leaf apex, but individuals feeding by the petiole would benefit the colony by providing an early-warning system via alarm signalling (Keiser et al., 2013). For a greater range of aphid and plant species, it would be interesting if empirical data on preferred aphid locations and ladybird searching tendencies could be used for modelling work to predict what proportion of the time ladybirds are likely to be on track to make contact with aphid prey. If it is true that ladybirds have a tendency to explore the edges and veins of leaf structures and if it were found that aphids tend not to occupy these areas, it would be interesting to consider why ladybirds might not have coevolved to search the areas of plants where they are most likely to encounter prey. Perhaps there is not enough of a selective pressure for this if their search strategy is successful enough times, particularly if aphids can be found in large numbers and/or on plant sections with relatively small surface areas such that the chance of successful predation is high regardless of the details of search tendency.

It is interesting that in most trials ladybirds did make contact with at least one aphid, and at least once, but only successfully captured prey in 13 of the 70 trials. Contact is thought to act as a cue for ladybirds to switch from extensive search to intensive search (Nakamuta, 1985, Ferran and Dixon, 1993), but here any switch that occurred in search behaviour certainly did not consistently improve a ladybird’s chances of successful predation. Aphids, particularly adults, commonly walked or ran away from the predator upon contact and, in the majority of trials, this was sufficient for them to evade capture. The four adults that were captured, however, were grabbed at the first contact between predator and prey and so did not have the opportunity to respond to untargeted contact by escaping. More quantitative explorations of the influence of a predator’s trajectory and/or the direction a prey individual is facing in a given predator-prey encounter on a prey individual’s propensity to exhibit antipredator defences, such as walking away or dropping, would be valuable in developing understanding of the likelihood of prey escape relative to successful predation. In this study, aphids were often contacted by a predator (sometimes they showed no reaction, sometimes they walked away, only rarely did they drop), but in many cases this was just a brushing of the predator past the aphid's legs rather than a targeted, forward-facing approach by the predator. In one trial on the lower side of a large elm leaf with 5 adult aphids, for example, a ladybird first brushed against the legs of one aphid while investigating some plant damage along the edge of the leaf – it did not seem to notice the aphid, and the aphid did not react. However, later, the same ladybird approached another adult aphid head-on, with the aphid facing its approach, and the aphid dropped immediately upon contact despite an absence of understructure below the leaf. Previous research suggests that the angle of approach of ladybirds can influence some aphid species' predator detection abilities and subsequent propensity to exhibit escape reactions (Dixon, 1958, Hajek and Dahlsten, 1987, Gish, 2021). More quantitative studies of pre-contact approach style by predators and associated evaluation of threat by aphids would be valuable.

On the theme of threat evaluation, it would also be interesting to uncover whether aphids can differentiate – and alter their antipredator behaviour accordingly – between ladybird predators that are actively hunting and likely to ultimately make a kill and those that are satiated or moving around plant structures primarily for other purposes. For example, walking ladybirds in the field have been observed frequently touching aphids without attacking or consuming them, a behaviour that has been attributed to the motivation of locomotion being a search for mates rather than prey (Honěk, 1985). Previous research into vertebrate prey species suggests that some prey will approach and 'inspect' their predators, in part so that they can assess the state (or motivation) of the predator (Magurran and Girling, 1986, Murphy and Pitcher, 1997) and/or the risk of imminent attack (Pitcher et al., 1986, Lima and Dill, 1990). The inspecting individuals' antipredator responses are flexible and can be adaptively selected based on the assessment of apparent risk. While predator inspection behaviour has received most attention in fish (Magurran, 1986, Pitcher et al., 1986, Lima and Dill, 1990, Dugatkin and Godin, 1992, Brown and Cowan, 2000, Walling et al., 2004, Dugatkin et al., 2005, Kent et al., 2019), a recent study has explored inspection of predator cues by invertebrates. Zaguri and Hawlena (2019) examined the reaction of the desert isopod *Hemilepistus reaumuri* to burrows deserted by one of their predators, the Israeli golden scorpion (*Scorpio palmatus*). As isopods did not closely inspect the area with predator cues on their first encounter with it (rather, they did so after seven days), this study suggests that the isopods implement risky inspection behaviours in order to validate whether the perceived threat is real (i.e. if a scorpion remains present), rather than to inform their defensive reactions. However, these findings are specific to these isopods and concern an environment where prey might benefit from planning their future spatial activity within a burrow system. Future investigations into the possibility of predator inspection by different invertebrates in the presence of predators in different states might shed more light on the flexibility of antipredator defences across predator-prey interactions at all scales. In the aphid-ladybird system, perhaps the frequency of different antipredator behaviours (evasive or aggressive) aphids exhibit, whether they approach ladybirds, and whether ladybirds successfully capture prey or not could be closely examined. From such data, it could be determined whether aphids flexibly alter their antipredator defences based on whether predators present were motivated to hunt (i.e. successfully made kills) or not. There might even be scope to explore whether aphids can detect, and subsequently flexibly alter their responses, whether ladybirds that are actively foraging have switched from extensive search behaviour to intensive search behaviour (Hassell and Southwood, 1978, Nakamuta, 1985, Ferran and Dixon, 1993), or vice versa.

Considering successful predation in this present study, the small sample size of captures by ladybirds limited conclusions that could be made about the speed of captures. This study did not explore the behaviour of ladybirds post-kill. More broadly, the number and proportion of aphids ladybirds contacted did appear to increase to some extent with the length of time ladybirds spent searching on a plant section. However, these explorations of the timing of both captures and discovery would be aided in future studies by including consideration of the surface area of plant sections. With bigger leaves, ladybirds could search for a long time and not encounter any aphids even if there were multiple present, while on smaller leaves it seemed to be less likely that ladybirds would not contact any aphids. Previous studies into ladybird foraging behaviour have made efforts to control for the effects of surface area (Carter et al., 1984, Clark and Messina, 1998, Francke et al., 2008, Reynolds and Cuddington, 2012), but future studies in the field might choose to investigate the variability of this factor within and between different plants as an influence on the likelihood of prey capture. As a final, predator-focussed point, the fact that ladybirds did not seem any more likely to successfully capture an aphid with increased experience of the trial format justifies the inclusion of all trials in the analyses without dividing results between ladybirds with different levels of experience.

Focussing more on the prey-perspective of this study, one key finding was that aphids rarely used dropping behaviour to escape from their current plant section. Dropping seemed to occur during a higher proportion of trials on nettle plants relative to the number of trials that took place on nettle overall, compared to on the other host plants. A previous study by Matsubara and Sugiura (2018) explored dropping by beetles from several host plants that varied in leaf shape in order to test the hypothesis that large, oval leaves can act as ‘safety nets’ for insects dropping from overlying leaves. They found that beetles on lower leaves of species with clefts in their underlying leaves frequently dropped onto the ground following a simulated attack, while beetles on the lower leaves of species with larger oval underlying leaves landed and re-clung onto those underlying leaves of the same plant. From these findings, Matsubara and Sugiura (2018) predict that beetles might change their antipredator behaviour from dropping to other defences, or avoid lower surfaces of leaves, when on species with clefts in their leaves, as opposed to when on plants with large, oval leaves. In Chapter Six, I found that post-drop re-clinging (or ‘instant recovery’) by potato aphids occurred more commonly when drops occurred from taller plants, a finding that was attributed to taller plants likely having grown more outwards as well as upwards and therefore presenting greater underlying foliage cover for dropped individuals. Plant height and foliage cover have also previously been reported as being important factors in the re-clinging likelihood of pea aphids (Meresman et al., 2017). In this present study, it might be the case that dropping is less adaptive and therefore less likely to occur in aphid species that have coevolved with plants that are less likely to have understructure below sections that aphids might drop from (and/or plants that do not tend to grow in groups of many members of the same genera). Additional observations of aphids reacting defensively to predators in the wild are necessary to support this hypothesis, though. Future studies should also aim to draw a quantitative distinction between understructure of the same plant species as the host or different plant species, though, as the propensity to drop may depend on the likelihood of re-clinging to a host plant specifically rather than any plant.

Regardless of re-clinging likelihood, host plant growth form might additionally influence the use of dropping by herbivorous prey. Recently, Matsubara and Sugiura (2021) compared the frequency of dropping by larvae and adults of many species of leaf beetle on woody versus herbaceous plants. The positioning of leaves is generally higher on woody plants than herbaceous plants, and so the costs of dropping to the ground and subsequently returning to a feeding position may be higher for beetles feeding on woody plants compared to herbaceous plants. As predicted, larvae feeding on woody plants were less likely to drop than those on herbaceous plants, but the drop rates of the flight-capable adults did not significantly differ – this is because dropping is less costly when rapid return by flying ahead of landing on the ground is possible (Matsubara and Sugiura, 2021). Similarly, dropping may be used less frequently by species of aphid that have primarily coevolved with woody host plants rather than herbaceous host plants, but further behavioural and phylogenetic studies are needed to clarify this.

It is also likely that dropping propensity is influenced by how efficiently an aphids' predator can forage on a given plant species' characteristics, such as architecture. On the one hand, the gross morphology and features of some plants have been found to impede predator movements, cause predators to fall, or in some other way make prey difficult to capture (Carter et al., 1984, Kareiva and Sahakian, 1990, Grevstad and Klepetka, 1992, Kauffman and Laroche, 1994, Clark and Messina, 1998, Legrand and Barbosa, 2003, Reynolds and Cuddington, 2012). However, such hinderances can be specific to predator instar, predator species, and/or the parts of plants specific species of prey colonise (Evans, 1976b, Carter et al., 1984). For example, Gurney and Hussey (1970) found that while larvae of the coccinellid species *Coelomegilla maculata* de G. tend to fall from the leaves of cucumber after being irritated by its glandular hairs, the larvae of two other coccinellid species were not deterred by the hairs. While it is reasonable to assume, from an evolutionary standpoint, that plants should have evolved their architecture and surface qualities as protection from environmental pressures and the herbivores they have coevolved with, those plants have also coevolved alongside the natural enemies of those herbivores. On the other hand, then, plants can also be expected to have developed adaptations to help rather than hinder predators' foraging (Grevstad and Klepetka, 1992). For example, Grevstad and Klepetka (1992) found that the detrimental effects of smooth leaf surfaces on the ability of several coccinellid predators to manoeuvre on the crucifer *Brassica campestris* is ameliorated to some extent by its complex structure, many edges and fewer flat surfaces; here plant morphology reduces predator falling rates, creating an accessible venue for successful predation. More recently, work by Nell and Mooney (2019) suggests that the structural complexity of some plants may limit birds' foraging abilities, increasing the survival (rather than specifically the foraging) of their arthropod prey, and indirectly increasing the ability of predatory arthropod populations to control their herbivore prey. Additionally, there is increasing evidence that plants synthesise protective chemicals in response to damage by herbivores that can serve as attractants to predators (Rhoades, 1985, Dicke and Sabelis, 1987, Schaller and Nentwig, 2000). These so-called ‘infochemicals’ (Vet and Dicke, 1992) can act as long-range cues that help predators locate patches of prey on plants (Han and Chen, 2002, Norkute et al., 2020), with some studies suggesting that plant-released volatiles can be specific enough to attract only predators that feed on the specific herbivore species causing damage (Yoon et al., 2010) or on the specific life stage of the herbivore prey present (Yoneya et al., 2009). Future studies evaluating the coevolutionary tri-trophic relationships between plants, aphids, and ladybirds might uncover instances where certain aphid species have evolved to react far less to perceived threat due to traits of their host plant essentially providing refuges or, conversely, react far more sensitively due to the host plant facilitating one or both of greater predator movement and prey detectability. To develop a fuller understanding of the behaviours and ecology at play, work endeavouring to explore the interactions between any two of these trophic levels should aim to also factor in the likely influence of the third trophic level wherever possible.

In this study, a greater proportion of adult aphids were contacted by ladybirds during trials, and most drops involved adults rather than nymphs. The slightly greater incidence of post-contact drops in this study might indicate that dropping is costly and that aphids tend to be risk-averse, waiting to drop until the sense of threat is imminent (i.e. contact is made). This may be even more so the case for nymphs, which are more vulnerable to desiccation and other risks associated with leaving the host plant (Losey and Denno, 1998a), resulting in them dropping very rarely even after being contacted by ladybirds. A number of previous studies with different aphid species have reported this reluctance of immature individuals to drop compared to adults (Montgomery and Nault, 1978, Roitberg and Myers, 1978, McConnell and Kring, 1990, Losey and Denno, 1998a), with their inability to relocate host plants and susceptibility to desiccation often proposed as explanations (Roitberg and Myers, 1978, Roitberg and Myers, 1979, Dill et al., 1990). Larval beetles have also previously been found to drop less frequently from plants than adults, and post-dropping return times are longer for larvae (Matsubara and Sugiura, 2018). However, with so few cases of dropping in this study it is difficult to draw any solid conclusions concerning the propensity to use dropping pre-relative to post-contact or between adults and nymphs. Certainly, dropping seems rare, and aphids were far more likely to walk or run away to evade predation during trials. Perhaps this was in part due to most trial situations taking place on tall, woody plants, and/or having absent or sparse, as opposed to dense, vegetation underlying the plant sections of interest.

Finally, weather conditions were not found to have any significant influence on the likelihood of dropping in this study. Previous work indicates that pea aphids ‘assess’ the risk of dropping and are less likely to leave host plants when their environment is hot and dry (Dill et al., 1990). Further, in Chapter Six I found that temperature might also be an important influencer of post-dropping behaviours in potato aphids, correlating negatively with duration of tonic immobility exhibited and positively with recovery rate back to plants; a finding that made sense given aphids’ vulnerability to desiccation (Broadbent and Hollings, 1951, Ruth et al., 1975, Roitberg and Myers, 1979). The lack of findings in this current work are likely because, firstly, dropping occurred only rarely and, secondly, the weather conditions were uncontrolled and did not vary dramatically over the ten days in which trials took place. Certainly, some weather conditions should be expected to be pervasive environmental stressors to insect herbivores, such as aphids, selecting for adaptive behaviours over evolutionary time. For example, the movement patterns and micro-site selection of some lepidopteran larvae and aphid species have been found to be significantly influenced by exposure to even moderate wind speeds (Leonard et al., 2016, Devegili et al., 2019), and pea aphids appear to adopt anticipatory and reactive crouching postures that reduce the likelihood of dislodgement from wind gusts (Ben-Ari et al., 2014). To better understand the influence of various climatic factors on aphids’ assessment of risk and propensity to drop, future studies should measure all environmental variables on-site and, ideally, consider microclimates (i.e. differences in temperature and exposure to wind and rain both at aphids’ original location on plants and at underlying points where the aphids are likely to land if they drop). Weather conditions may also influence post-dropping behaviours (discussed in greater detail in Chapter Six), for example recent rain might increase the moisture content of the underlying substrate and make time spent off-plant less risky in terms of the threat of desiccation. There have also been suggestions that different weather conditions can affect the foraging behaviour of predators, for example, windy conditions and related increased plant movement can negatively affect the hunting performance of ladybirds (Barton, 2014). Behavioural assays of predator-prey interactions should be conducted on different wild plants at different times of day and during different seasons in order to build a bank of data including great variation in climatic and weather conditions.

# Chapter 8: Orientation and substrate influence apparent turning biases by the 7-spot ladybird, *Coccinella septempunctata*

Material from this chapter formed the basis of the publication: Humphreys, R. K. & Ruxton, G. D. 2020. Do orientation and substrate influence apparent turning biases by the 7-spot ladybird, *Coccinella septempunctata*? Behaviour, 157(3-4), pp.205-230.

How foraging predators explore their environment is an important aspect of predator-prey interactions. Girling et al. (2007) tested *Coccinella septempunctata* in a Y-maze, finding that approximately 45% of individuals displayed significant turning biases. I extend the work of Girling et al. by testing for turning bias on vertical as well as horizontal structures, and on natural Y-shaped twigs as well as artificial twigs. No significant patterns of ‘handedness’ were apparent with vertical orientation, on either substrate. With horizontal orientation (as used by Girling et al.), significant turning biases were exhibited on artificial but not natural twigs. Overall, although turning biases are theoretically efficient when searching branched structures, I propose that in natural environments ladybirds will base their foraging movements on environmental cues (something I will explore further in Chapter Nine).

## 8.1 Introduction

The temporal and spatial dynamics of predator-prey interactions are important to the suppression of prey by predators, and so will hold significance for the fitness of both prey and predators at population scales. An important characteristic of predators that can directly influence these dynamics is the utilisation of particular strategies when searching for prey. Smith (1974) defined a search strategy as “a set of basic rules of scanning and locomotion which results in the effective encountering of a specific distribution of food” and a search tactic as “an adaptive change in scanning or locomotion occurring once a predator has arrived in a specific area where prey are available”. Given that an organism’s evolutionary fitness depends on its ability to optimise both the quantity and quality of its diet, search strategies and search tactics should be expected to be under strong natural selection to maximise intake while minimising costs (Hassell and Southwood, 1978), including such relevant costs as time and energy expended. The spatial structure of an environment is one factor that will influence the efficiency of search and this is particularly true for predatory insects (Hassell and Southwood, 1978). Coccinellids have often been the focus of studies on foraging behaviour, but many of these studies utilise the larvae rather than adults (Ferran and Dixon, 1993, Pervez and Yadav, 2018). Further exploration of adult foraging behaviour can be expected to shed light on how these predators maximise their fitness when searching for prey.

It is reasonable to predict that in branched plant environments, coccinellids will be under strong selection to use the most efficient mechanism to locate prey distributed in patches across a plant (Girling et al., 2007). Research suggests that plant architecture can significantly affect the foraging efficiency of ladybirds (Carter et al., 1984, Grevstad and Klepetka, 1992, Clark and Messina, 1998), with highly branched plants potentially facilitating greater ease of movement for predators (Reynolds and Cuddington, 2012). The sensory abilities of ladybirds are, at present, poorly understood (Ferran and Dixon, 1993, Pervez and Yadav, 2018), but while visual (Nakamuta, 1984, Harmon et al., 1998, Lim and Ben-Yakir, 2020) and chemical volatile (Ninkovic et al., 2001, Pettersson et al., 2005, Pettersson et al., 2008, Norkute et al., 2020) cues may play some role in prey detection, at least for some species, some suggest that they might only be used over short distances (Dixon, 2000). The most efficient rules for locomotion as part of extensive search, before encountering a prey patch and switching to intensive search (Hassell and Southwood, 1978), therefore should be selected in order to maximise an individual's chances of encountering prey patches. Here my focus is on this extensive search, that is, how do ladybirds forage on plant structures ahead of encountering any prey?

Girling et al. (2007) investigated the hypothesis that seven-spot ladybird (*Coccinella septempunctata*, Coleoptera: Coccinellidae) adults exhibit turning biases in a way that improves their searching efficiency when foraging in complex branching environments. This hypothesis stemmed from research into ants' 'outline-tracing', a search strategy that is considered to be the most efficient on unfamiliar branched structures  in terms of costs and effectiveness (Jander, 1990). Outline tracing is where a foraging individual arriving at branching points will consistently choose the left or the right option and is therefore based on an insect demonstrating an innate bias to turn in one direction over another; this is referred to as 'handedness' (Girling et al., 2007). In theory, in the absence of other cues of prey location, outline-tracing cannot be improved upon as an arboreal search strategy for exploring a complex branching environment (Jander, 1990). Thus, using a Y-maze (as is typical for experiments on foraging behaviour), Girling et al. sought to test whether ladybirds show significant turning biases. They also estimated the searching efficiencies of ladybirds with various turning biases using a simulation model. Their findings suggested that 45% of tested individuals demonstrated significant turning biases and that simulated foraging benefits increased with the degree of bias implemented by individuals (Girling et al., 2007).

Further support for the existence of individual handedness has since been reported for many invertebrates (Frasnelli, 2013, Frasnelli, 2017), including ants (Hunt et al., 2014, Endlein and Sitti, 2018), bees (Ong et al., 2017), and giant water bugs *Belostoma flumineum* Say (Heteroptera: Belostomatidae) (Kight et al., 2008). Bell and Niven (2014) reported an interesting context-dependent handedness in desert locusts (*Schistocerca gregaria*). Locusts exhibited handedness during targeted forelimb placement but not whilst walking, reminiscent of the context-dependent handedness of some vertebrates' handedness, including humans. Such handedness may reduce the computations involved in forelimb selection for targeted movements and thus be adaptive, enhancing the efficiency of the nervous system (Levy, 1977, Bell and Niven, 2014). Recently, O'Shea-Wheller (2019) reported further evidence for invertebrate context-dependent handedness, finding that foraging honey bees (*Apis mellifera*) exhibit a strong rightward turning bias, accompanied by reduced decision latency when entering open cavities. However, they show no directional preference in sequential choice-mazes, where thigmotaxis instead predominates. Honeybee workers are thought to be better equipped for sensory investigation and threat response using their right antenna and eye, so this turning bias should be expected to be adaptive in the context of exploring open cavities (O'Shea-Wheller, 2019).

Lateralisation and 'handedness' of behaviours is also widespread in vertebrates (see Güntürkün [2012] and references therein and Maciejewska et al. [2016] for an example). Left-right asymmetries in the brains (or nervous systems) and behaviour of vertebrates and invertebrates are theorised to increase individual efficiency by: avoiding duplication of functions (Levy, 1977), enabling parallel processing of information (Rogers et al., 2004), and by allowing one 'side' of the nervous system or brain hemisphere to control actions and thus prevent the simultaneous initiation of incompatible responses (Vallortigara, 2000, Ghirlanda et al., 2009). Indeed, often animals that show lateralised behaviours, such as turning biases, seem to outperform those that do not in many circumstances (McGrew and Marchant, 1999, Güntürkün et al., 2000, Rogers et al., 2004). Handedness, therefore, may be predicted to be useful for coccinellids – alongside other animals – both as an efficient way to forage on structures in particular contexts and as an example of lateralisation that could improve the efficiency of cognition.

However, no studies have yet attempted explore the generality of Girling et al.'s (2007) findings or begin to consider the possibility of context-dependent handedness in ladybirds. This current study sought to explore the generality of turning biases in ladybirds further by extending the methodology used by Girling et al. (2007) in several ways. Firstly, I explored whether the orientation of a Y-choice-presenting set-up would influence apparent turning bias. Girling et al. (2007) presented their Y-maze horizontally so that they could directly compare their findings with the majority of other studies (that positioned similar equipment horizontally). However, Bansch (1966) found no evidence of handedness in two-spot ladybirds (*Adalia bipunctata*) when searching a 50cm-high branching model tree and suggested that geotaxis was instead the salient control of behaviour. Thus, there is reason to expect that results of horizontal choice tests may not necessarily transfer to branched structures with a vertical orientation. Ladybirds can be found foraging on a very wide variety of plants with branches orientated at a great diversity of inclinations, so investigating their foraging behaviours on structures angled differently to 180 degrees could be insightful. Secondly, I compared the potential influence of substrate on turning bias, by testing ladybirds on natural and artificially-constructed twigs. Although Girling et al. (2007) improved on previous studies by including a linear wire in their Y-tube olfactometer for ladybirds to follow along, resembling the branch of a plant, this substrate may be unfamiliar to their subjects in its tactile properties. The exploration of turning bias in wild ladybird populations was here extended by comparing movements on Y-shaped twigs acquired from ladybirds' natural habitat with those on artificial wire twigs constructed to similar lengths. Finally, I investigated turning directions by considering both whether the selected branches were the designated 'left' or 'right' branch of twigs and whether ladybirds' selected branches were 'left' or 'right' from the perspective of the ladybird as they approached the turning decision point. I arbitrarily designated one physical branch as left and right for each twig (as Girling et al. had pre-determined 'lefts' and 'rights' for their constructed wires) and recorded both the physical branch selected and the 'ladybird's perspective' branch selected. The second approach accounted for occasions where the ladybirds may have circled round the stem of the twig before making a turning decision. That is, two ladybirds could select the same physical branch and this could be recorded as a left choice from one's perspective and a right choice from the other's perspective – if they approached the junction from opposite sides of the main starting stem (see Figure 8.1). I considered the 'observer reference frame' approach to give my work commonality with Girling et al., but also introduced the 'ladybird perspective' approach since this more naturally reflects the behavioural processes of the ladybirds.

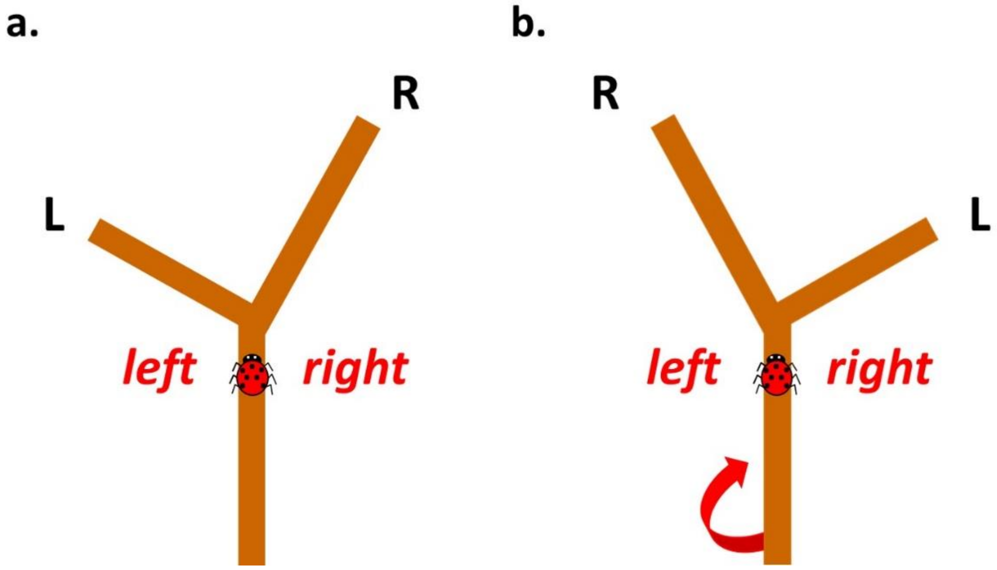


**Figure 8. 1:** An example of how a ladybird's movement around the other side of a twig and subsequent approach to the decision point would affect the turning decision from the ladybird's perspective. Both images depict the same theoretical Y-shaped twig, with its longer branch designated as 'R' (right) and its shorter branch designated as 'L' (left). In situation **a)** the ladybird remains on the 'front' side of the twig and so the turning choices from its perspective (italicised lower-case) match those presented by the designated branch labels ('observer reference frame' approach). In situation **b)** however, the ladybird is approaching those same physical branches on the twig, but has moved around the 'back' of the twig, such that the turning choices from its perspective no longer match those presented by the designated branch labels.

## 8.2 Materials and methods

### 8.2.1 Study organisms

Adult seven-spot ladybirds were collected along Lade Braes walk, St Andrews (LAT: 56.34, LONG: -2.81) during August 2019. Ladybirds were kept individually in sterilised plastic tubs (4.8 cm [H] x 6.5 cm [D]) with a nylon mesh lid for ventilation. Ladybirds were transferred individually using a fine-haired paintbrush so as to minimise stress and injury during handling. Each container  was provided with several small twigs taken from the original habitat, a piece of damp paper towel, and raisins as a food supplement, which were replaced daily. Ladybirds could not be starved 24 hours ahead of trials (as per Girling et al., 2007) because trials for any individual ladybird took place over several days. Although raisins were provided to help keep ladybirds alive over this time, aphids are the primary dietary staple for ladybirds and so the raisins were not expected to diminish ladybirds' foraging behaviour when tested. In any case, ladybirds were rarely seen to settle on raisins when in their containers and little damage was found on replaced raisins, suggesting that ladybirds will have been hungry or even starved ahead of testing and therefore highly motivated to forage. Room temperature was kept at ~18°C via a thermostat heating system and the tubs were kept by a window so that ladybirds had access to natural light cycles even when not participating in testing. No ladybird was kept for more than three days before being released.

### 8.2.2 Design of experimental set-up

Seven Y-shaped twigs were collected along Lade Braes walk, St Andrews (LAT: 56.34, LONG: -2.81). The twigs varied in height, girth, and texture, providing a range of natural variation to test the subjects on (Figure 8.2). Each twig was assigned a number and had the branches leading from the stem of the 'Y' randomly designated as 'left' and 'right'; the left branch was marked with a tiny piece of gold tape. Seven artificial Y-shaped twigs were then constructed from plastic-coated garden wire, each matching to within 1cm the lengths of each of the branches of one of the natural twigs (see Figure 8.3 for examples). For each trial, depending on whether a natural or artificial twig was required, a twig of that given substrate was selected randomly.

For each trial, the selected twig was affixed by blu tack in a large, sterilised clear plastic container: 20 cm (H) x 32 cm (W) x 23 cm (D). In experiment 1, the twig was orientated vertically, affixed to the centre of the base of the container such that the left-right axis of the twig was aligned with the widest length of the container (see Figure 8.4a-b). In experiment 2, the twig was orientated horizontally, affixed to the centre of one of the short wall sides of the container such that the left-right axis of the twig was aligned with the short wall but that the Y pointed into the centre of the container (see Figure 8.4c-d).

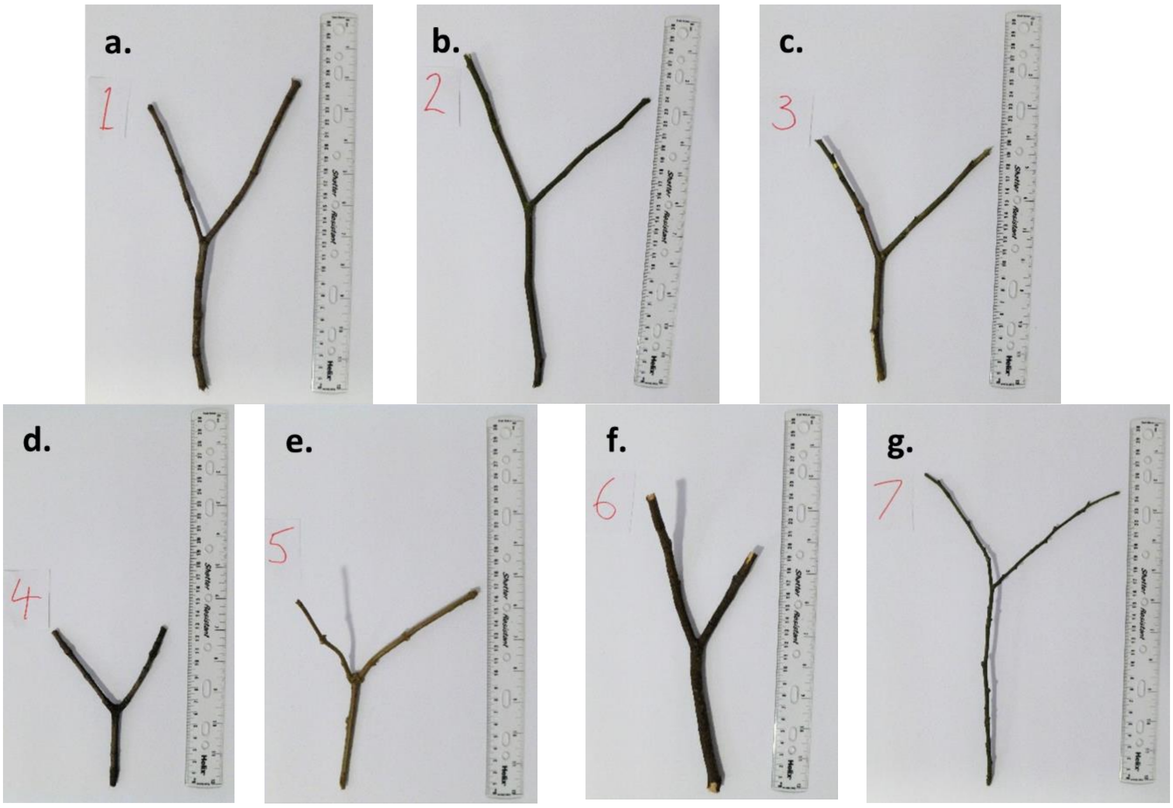


**Figure 8. 2:** The seven natural Y-shaped twigs used in both experiments 1 and 2, each alongside a 30cm ruler (**a-g** corresponding with the twig numbers 1-7).

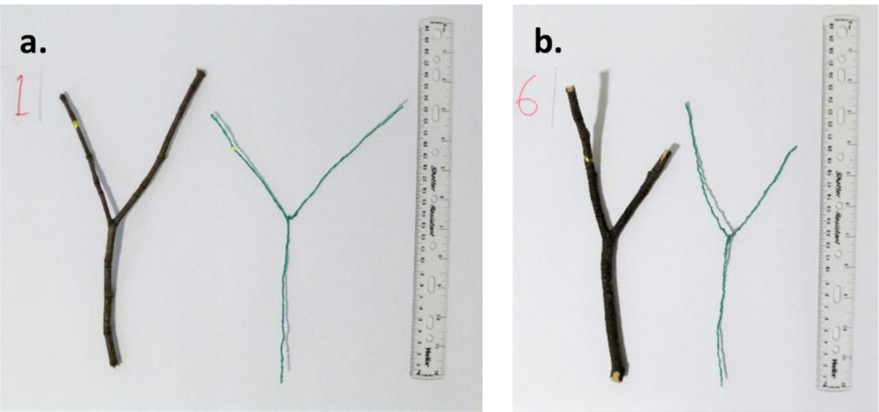


**Figure 8. 3: a)** The corresponding natural and artificial '1' twigs side-by-side alongside a 30cm ruler. **b)** The corresponding natural and artificial '6' twigs side-by-side alongside a 30cm ruler.

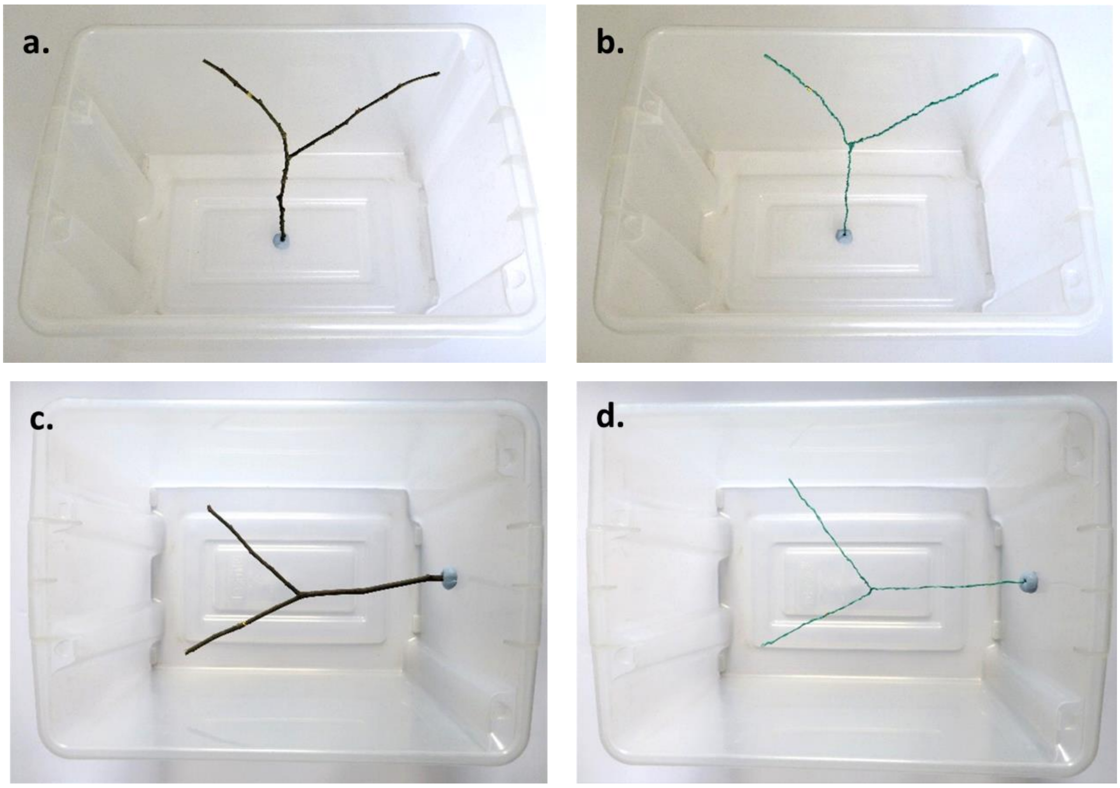


**Figure 8. 4: a)** Natural twig '7' oriented vertically for a trial. **b)** Artificial twig '7' oriented vertically for a trial. **c)** Directly overhead view of natural twig '2' oriented horizontally for a trial. **d)** Directly overhead view of artificial twig '2' oriented horizontally for a trial.

### 8.2.3 Test for bias in individuals

In total, eighteen individual ladybirds were tested over the whole study. For each experiment, nine individual ladybirds were tested across three days, running blocks of five trials per individual with natural twigs and five trials with artificial twigs on each day. Individuals experienced trials with both twig substrates in the morning (7:30 am – 12:00 pm) and in the afternoon (1:30 pm – 6 pm) such that the order of testing was not the same on every day. The order of testing for the ladybird individuals was randomised at the beginning of each five-trial block. Upon selection, ladybirds were transferred to a different plastic container (4cm [H] x 9cm [W] x 9cm [D]) using the paintbrush and were kept in this container between each of their trials. In total, each ladybird made 15 choice decisions on randomly-selected natural twigs and 15 on artificial ones. The relatively small sample size per experiment (n = 9) was seen as an unfortunate but necessary trade-off in order to run 30 trials with each individual, at different times of day and over several days, while still completing the overall study within the same short time period in the season to avoid developmental and seasonal effects becoming confounders.

At the beginning of a trial, an audio recording which ran a timer was started, ready for behaviours to be dictated to it as they occurred and for the timing of events around the movements made to be kept track of as the trial took place. The ladybird was then introduced to the base of the twig in the middle of the main stem from the ‘front’ of the twig (with the designated branches presented left to right) using the paintbrush. The ladybird was allowed to run up and down the paintbrush (to overcome handling stress) until it opted to move onto the base of the twig. Once on the base of the twig, a ladybird was given a maximum of two minutes to move up the main stem and move a minimum of 1cm up one of the branches. Once this had occurred, the individual was considered as having made a turning decision and whether the selected branch was the designated left or right branch of the twig was recorded (the ‘observer reference frame approach). Whether the selected branch was left or right from the perspective of the ladybird as it approached the turning decision point was also recorded, in order to account for occasions where the ladybird had moved around to the other side of the twig stem before making a turning decision (the ‘ladybird perspective’ approach).

If ladybirds left the twig before reaching the branching point and making a decision, the trial was re-started. The twig branches never touched and were never in reach of the sides of the plastic container. During all trials, I remained at a distance, to prevent breath from influencing behaviour, and avoided casting a shadow over the equipment. As discussed in the introduction, Dixon (2000) suggested that ladybird sensory systems are attuned to short spatial scales, considerably shorter than the distance between myself and the set-up. While I cannot be certain of whether my presence influenced visual cues, I did not systematically vary my movement or my proximity to the apparatus. I was also, of course, far larger than potential predators of ladybirds would be whilst attacking and so was unlikely to have provoked responses more associated with avoiding predation than foraging.  The room was well-lit by natural light, and after each trial the trial container was rotated by 45° to control for the effects of any environmental gradients within the room. Unlike in the study by Girling et al. (2007), neither the natural nor the artificial twigs were cleaned between trials. Although I acknowledge that ladybirds may leave behind chemical residues on substrates they explore, I know of no evidence of ladybird sensitivity to conspecific residues when foraging. Further, the structures ladybirds encounter in the wild would likely be covered in residues of other organisms that have explored the plant. Cleaning down the natural substrates may have significantly impacted some of the natural traits I sought to explore the influence of, removing residues from the wild and potentially damaging the bark. It would then be unhelpful to the comparison aspect of this study to clean down the artificial substrates. The lack of cleaning procedure did not result in ladybirds either consistently following or avoiding the path of the previous ladybird on any given twig – artificial or natural.

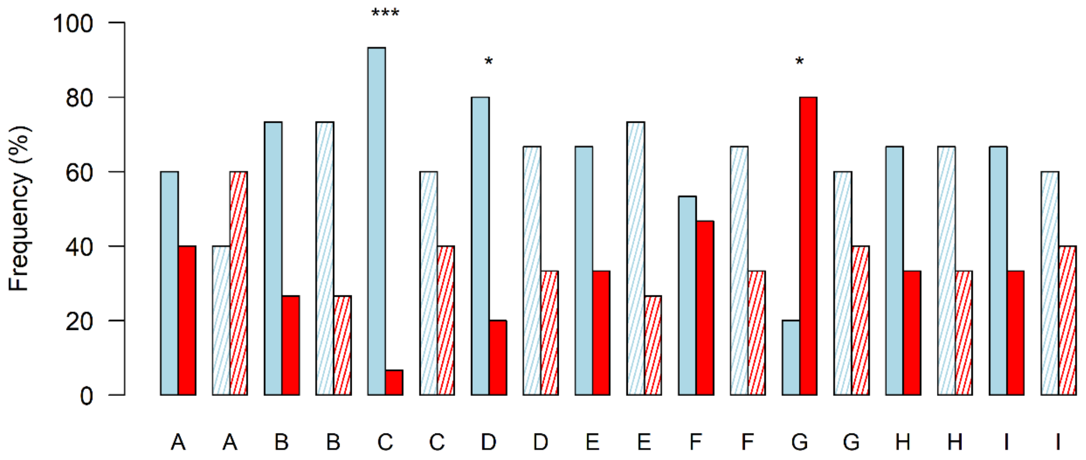
### 8.2.4 Analysis

All analyses were conducted using R version 3.4.3 (R Core Team, 2017). For all comparisons of observed frequencies of left-or-right turning decisions in each experiment, chi-square tests were used. GLMs containing only turning choice as a factor also confirmed the findings of chi-square tests comparing the frequency of left-or-right turning decisions.

## 8.3 Results

### 8.3.1 Experiment 1: Vertical orientation

Looking at the turning decisions of individuals on vertically-orientated twigs (Figure 8.5a), on natural twigs one third of individuals (three out of nine) showed significant differences in chi-square tests concerning their selection of left-or-right across their 15 choices. In all of these cases the biases were only apparent from the designated branch labels rather than being left-or-right biases from the ladybird's perspective as they approached the decision point. As each individual underwent multiple trials separately, a number of individuals should be expected to produce positive results even in the absence of any turning bias simply through type I errors. Ten thousand replicate simulations of the experiment were conducted under the assumption of all individuals having no bias, in order to test the likelihood of these results in a situation of no true individual-level variation. Of these simulations, less than 1% produced three or more out of nine individuals with apparent turning biases through type I error alone, suggesting that the preferences shown in terms of the twigs' designated left or right branches were not simply down to type I errors. However, since there was no evidence of left-or-right individual biases from a ladybird's perspective (Figure 8.5a), the most parsimonious explanation is that some ladybirds did not choose branches randomly but rather selected according to some physical twig trait that was not equally balanced across branches designated left or right on the twigs that they encountered. That is, I can tentatively suggest that some ladybirds do not choose their path randomly but do not use left-or-right bias to make their non-random choice; rather, some unidentified physical trait (or traits) of the natural substrate influence turning choice. Such physical twig traits could include: the colouration, textures, patterning of the twig bark, thickness, and the structure of the branched turning choice.


**a. Vertical choice: natural**

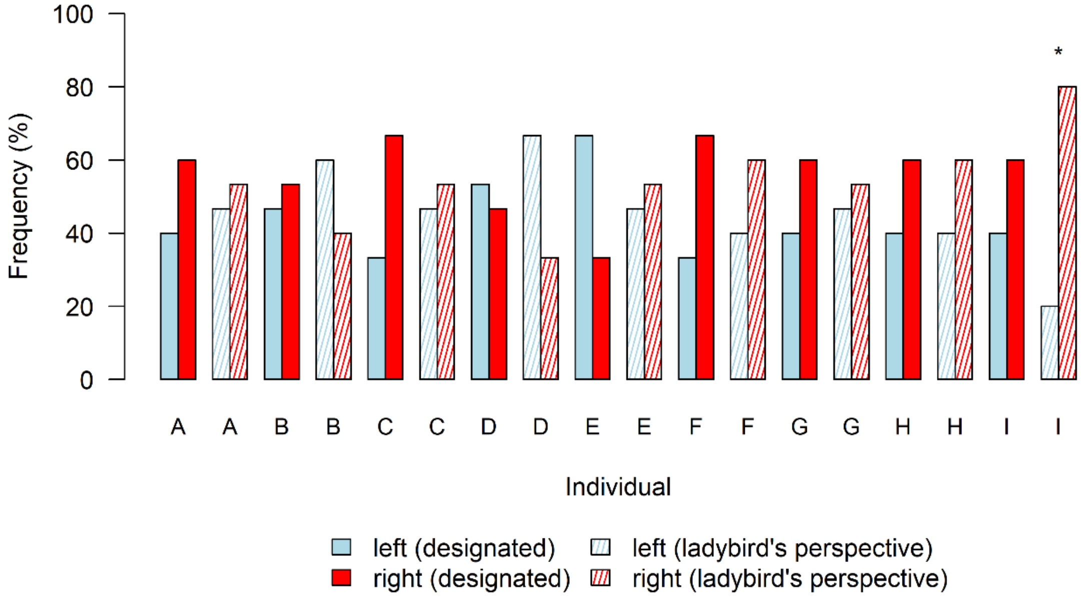

**b. Vertical choice: artificial**

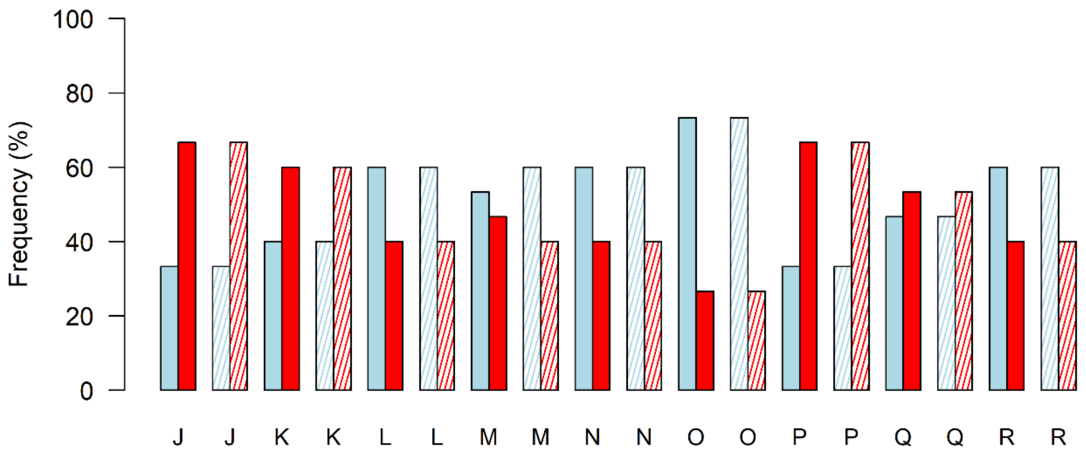
**Figure 8. 5:** The % times individual ladybirds chose to turn left (pale blue) or right (dark red) on: **a)** natural and **b)** artificial twigs across experiment 1, from both the perspective of the designated branches (‘designated’, filled bars) and the perspective of the ladybird as it reached the turning point (‘ladybird’s perspective’, shaded bars). Asterisks indicate the chi-square test significance of the frequency of left and right decisions by different individuals, where *<0.05, and ***<0.001.

Across the artificial twigs (Figure 8.5b), only one of the nine individuals showed significant differences in chi-square tests of their left-or-right decisions, and in this case the bias was only significant as turning decisions made from the ladybird’s perspective, as opposed to the designated branches. 28% of 10,000 simulations assuming all ladybirds were unbiased produced at least as extreme an occurrence of turning bias though type I errors alone. From this, there is no strong evidence to suggest consistent bias for left or right in these ladybirds on this vertically-orientated artificial substrate.

### 8.3.2 Experiment 2: Horizontal orientation

Looking at the turning decisions of individuals on horizontally-orientated twigs, on natural twigs no individuals showed any significant differences in chi-square tests of the frequencies of left and right decisions, both based on branch designations and the ladybird’s perspective when approaching the turning point (Figure 8.6a). On the artificial twigs, however, four of the nine individuals showed significant turning tendencies (Figure 8.6b). Re-sampling simulations of 10,000 runs found that less than 0.1% produced four or more out of nine individuals with turning biases through type I error alone, suggesting that the individual preferences shown in terms of the twigs’ designated left or right branches were real. Individuals L, M, Q and R chose the designated left branch significantly more times than the designated right branch (Figure 8.6b). Four of the nine individuals tested in experiment 2 (44.4%), therefore, displayed significant turning biases onto the left designated branches, resembling the 45% of individuals Girling et al. (2007) found to exhibit significant turning biases (19 out of 42). However, in this current study only individuals M and R exhibited this bias to a significant degree ($P < 0.01$) from the ladybirds’ perspective at the decision point (Figure 8.6b). Re-sampling simulations found that of 10,000 runs 4% produced two or more out of nine individuals with turning biases through type I error alone, suggesting that the preferences in terms of ladybirds’ approach choice was likely down to individuals’ bias. Two of the nine individuals tested in experiment 2 (22.2%), therefore, displayed significant turning biases to the left, but this was a smaller proportion of ladybirds than the 19 out of 42 (45%) individuals Girling et al. (2007) found to exhibit significant turning biases in their study also using a horizontally-orientated artificial substrate.

a. Horizontal choice: natural



b. Horizontal choice: artificial

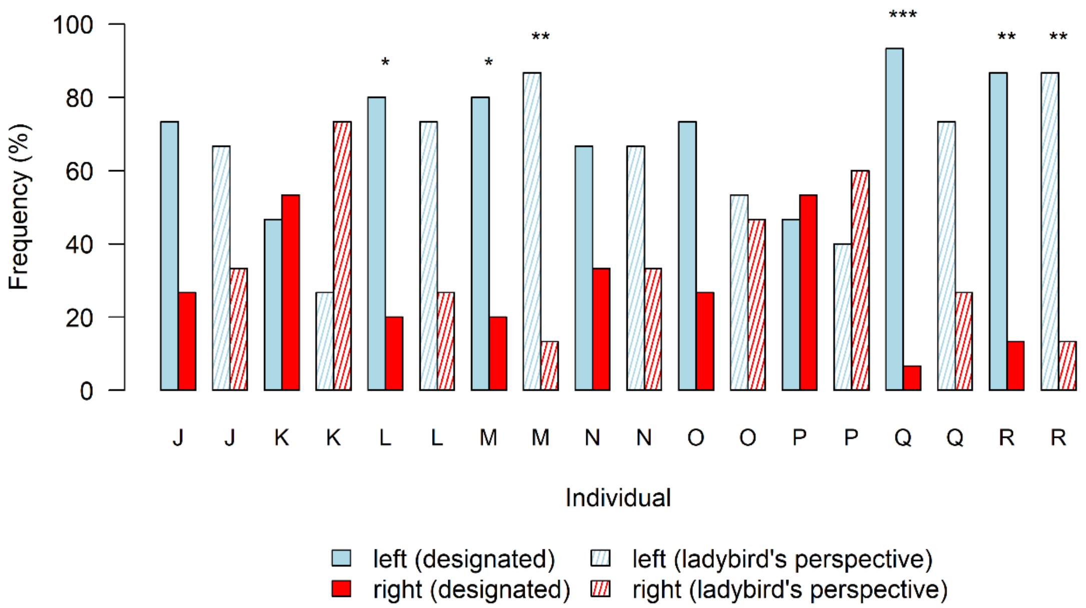


**Figure 8. 6:** The % times individual ladybirds chose to turn left (pale blue) or right (dark red) on: **a)** natural and **b)** artificial twigs across experiment 2, from both the perspective of the designated branches (‘designated’, filled bars) and the perspective of the ladybird as it reached the turning point (‘ladybird’s perspective’, shaded bars). Asterisks indicate the chi-square test significance of the frequency of left and right decisions by different individuals, where *<0.05, **<0.01, and ***<0.001.

### 8.3.3 Choices made from different perspectives

Examining how many times ladybirds’ turning choice as they approached the decision point corresponded with the pre-designated ‘left’ or ‘right’ labels of twigs (Table 8.1), it is clear that ladybirds moved around the other side of the branch far less frequently during trials with horizontally-orientated twigs than during trials with vertically-orientated twigs. When the set-up was vertical, ladybirds significantly more frequently made their choice from the ‘front’ of the twig – such that their choice matched the designated branch choice – when on natural twigs ($\chi^2_1 = 3.9$, $P < 0.05$), but this was not the case on artificial twigs ($\chi^2_1 = 0.007$, $P = 0.931$). When the set-up was horizontal, ladybirds again made more choices from the ‘front’ of natural twigs than on artificial twigs (Table 8.1), but across both substrate types far more ladybirds made their decision from the ‘front’ of twigs, matching the designated branch choice, than around the back (natural: $\chi^2_1 = 131.0$, $P < 0.001$, artificial: $\chi^2_1 = 58.7$, $P < 0.001$). Aggregating the data on matching versus differing choices, it is clear that there was a stronger tendency for ladybirds’ turning decisions to match rather than differ with the choice of designated branch on horizontal twigs ($\chi^2_1 = 182.5$, $P < 0.001$) regardless of substrate than on vertical twigs ($\chi^2_1 = 2.1$, $P = 0.144$). Essentially, when on horizontally-orientated branches, ladybirds strongly preferred to walk above than below the branch. This is to be expected, since walking above the branch causes gravity to act to secure them to the branch, whereas when walking underneath they must resist the force of gravity acting to detach them. On vertically-orientated branches there is no such strong preference, likely because orientation with respect to the branch would not affect gravitational forces experienced.

**Table 8. 1:** Frequency of trials where the left-right turning choice made from the ladybirds' perspective matched the designated ‘left’ or ‘right’ label assigned to the chosen branch for each combination of methodological set-up.

| **Set-up** | **Matching choices** | **Differing choices** | **Total** |
|---|---:|---:|---:|
| Vertical & natural | 79 | 56 | 135 |
| Vertical & artificial | 68 | 67 | 135 |
| Horizontal & natural | 134 | 1 | 135 |
| Horizontal & artificial | 112 | 23 | 135 |

## 8.4 Discussion

During experiment 1 (vertical orientation), in trials with the artificial twigs only one individual exhibited a significant turning bias during their trials, and this was from their perspective rather than being a bias for designated sides of twigs. However, this single incident of apparent handedness had a reasonable likelihood of resulting simply from type I error. On the natural twigs, however, three of the nine individuals in experiment 1 demonstrated significant differences in the frequencies of left and right choices they made in terms of the designated branches they selected, but none showed a significant turning bias towards either direction when considering the direction in which they turned from their approach. Together, these findings suggest that no real patterns of handedness were seen across either substrate in experiment 1. Perhaps, as in the study by Bansch (1966), geotaxis best explains the behaviour seen with the vertical experimental set-up. That is, rather than handedness influencing movement up vertically-orientated branched structures, ladybirds may have primarily sought to move against the force of gravity and head upwards regardless of junctures forcing turning decisions – the turning choices themselves may be irrelevant to the ladybirds' overriding goal of moving up the structure.

Turning then to experiment 2, the horizontal orientation of twigs resembled more closely the set-up of Girling et al. (2007) than experiment 1. Looking at individual turning choices across trials with natural twigs, no significant differences were seen across either the choices between designated branch labels or the turning choices from the individual's perspective for any of the ladybirds tested. It is important to note that throughout experiment 2 there was much less of a difference between the turning choice from the ladybirds' perspective and from the designated turning choices assigned to the branches than there was in experiment 1 (Table 8.1). With horizontally-orientated twigs, regardless of substrate, ladybirds showed a strong tendency to explore their environment while remaining on the top side of twigs as opposed to moving along below it where they would have to hang upside down. This suggests an influence of geotaxis on ladybird movement, as the difference felt in gravity will be greater in the choice between twig sides during horizontal orientation (i.e. one side involves being suspended upside down) than it is on either side of a vertically-orientated twig. In contrast to the natural twigs, when on horizontally-orientated artificial twigs in experiment 2, four of the nine ladybirds showed significant tendencies to select the designated left branch of twigs, but only two of these individuals also exhibited a significant bias in the decision to turn left from their perspective on the approach to the decision point. Interestingly, both cases of significant bias from the ladybird's perspective involved individuals turning predominately left. While the direction of bias in cases of handedness across different animals it sometimes found to be biased itself at the population level (Hunt et al., 2014, Frasnelli and Vallortigara, 2018, O'Shea-Wheller, 2019), Girling et al. (2007) reported that of the individuals they found to exhibit significant turning biases there were similar number of individuals biased to the left and right. The findings here suggest that when twigs are orientated horizontally, significant turning biases are more likely to be exhibited by individuals on artificial substrates than on natural substrates. However, anecdotally, throughout this present study it was far harder to get ladybirds to run along the horizontally-orientated twigs than the vertically-orientated twigs; far more trials had to be re-started as ladybirds would often leave the main stem before reaching the turning point to make a decision. This may suggest that ladybirds prefer to move along vertical branches than horizontal ones.

To conclude, when twigs were orientated vertically (experiment 1), little evidence was found for ‘handedness’ from the ladybirds’ perspective on either substrate. Twigs naturally occur at a diversity of angles, but phototaxic growth may tend to encourage upward orientation. This may mean that the findings from experiment 1 are, to some extent, more likely to reflect behaviours exhibited by ladybirds in most circumstances in the wild. When twigs were orientated horizontally (experiment 2), as they were in the original study of Girling et al., more individual turning biases were seen in terms of the observer reference frame on artificial substrate than on natural substrate, but only a small proportion of individuals exhibited this ‘handedness’ from their perspective when approaching the turning decision. Ladybirds might be somewhat more inclined to demonstrate innate turning bias on artificial twigs that are orientated horizontally, perhaps because the lack of gravitational or light variation leaves ladybirds with fewer natural cues on which to guide foraging movements. Concerning the differences in vertical and horizontal substrate handedness, it might also be the case that ladybirds show some context-dependent handedness (Bell and Niven, 2014, O'Shea-Wheller, 2019). In terms of searching for prey, it may be logical to have a bias for systematic search that involves handedness on one level of a plant structure, but the direction of turning choices may be unimportant as ladybirds move up onto higher levels. Further work on the possibility for context-dependent handedness in individual ladybirds could shed light on whether this occurs in the wild and, if so, how does it provide an adaptive function.

Predator-prey interactions are fundamentally impacted by the ways in which foraging predators explore their environment. Any search strategies or tactics employed by predators have the potential to significantly influence the fitness of prey and their predators at both individual and population scales. Theoretically, having a turning bias is a very effective search strategy for invertebrate predators in branched environments; this was demonstrated in simulations by Girling et al. (2007). However, while in the stimulus-free world of virtual coccinellids handedness may be the most efficient tool with which to locate prey patches, it is likely that in the real world there are natural cues that ladybirds could attend to that are better indicators of where they could successfully find prey and, therefore, following them is preferred as a foraging strategy compared to innate turning behaviour (something that will be explored in Chapter Nine). Ladybirds have been found to utilise both visual (Nakamuta, 1984, Harmon et al., 1998, Lim and Ben-Yakir, 2020) and olfactory (Ninkovic et al., 2001, Pettersson et al., 2005, Pettersson et al., 2008, Norkute et al., 2020) cues when foraging for prey, indicating that a variety of sensory modalities are important in guiding their behaviours. Perhaps ladybirds require an environment that presents the cues they might experience in the natural world in order to demonstrate normal foraging behaviours, such as: plant architecture, variable branch thickness, branch texture, gravity, and gradients in light and/or temperature. The recent study by O'Shea-Wheller (2019) found that foraging honey bees exhibit strong right turning bias when entering open cavities, but do not show this directional preference in sequential choice-mazes. I suggest that context may also be important in the demonstration of handedness by foraging ladybirds. My results confirm Girling et al.’s observation (2007) that ladybirds can show handedness in their navigational decisions. However, I suggest that Girling et al.’s experimental set-up may have provided a set of circumstances (a simple artificial substrate orientated horizontally) that denies ladybirds other cues as to likely prey locations and allows handedness to be exhibited. When I deviated from this situation (by using vertical orientation and/or natural substrate) then the ladybirds may have used cues – such as gravity, or natural twig textures and architecture – more familiar to them when foraging naturally to make turning decisions, and did not appear to display innate handedness.

It is also possible that ladybirds have not been strongly selected to search plants efficiently for prey, either by using turning biases or by the most effective use of natural cues. Ladybirds may be less time-constrained than optimal foraging theory implicitly assumes, perhaps in part due to them being highly chemically-defended (and thus at low risk of predation while foraging). Thus, it may be that we should not expect ladybirds to demonstrate ‘optimal’ behaviour in terms of efficiency when allowed to forage naturally. In fact, coccinellid larvae usually search randomly rather than systematically for prey, with the edges and veins of leaves often determining the pattern of movement and chance playing a large part in their encountering of aphids (Banks, 1957). Larvae have also been reported as wasting time and energy repeatedly visiting parts of plants which they have already visited (Banks, 1957).

Certainly, ladybirds appear to search more thoroughly once prey have been encountered, switching to intensive area-restricted search (Banks, 1957, Nakamuta, 1985, Ferran and Dixon, 1993), but the more extensive search may not follow as efficient rules as theory predicts. Further work on the foraging behaviour of adult ladybirds would do well to track foraging for aphids on real branched plants while monitoring differences in capture rates (Girling et al., 2007) alongside variability in both traits of the plants and broader environmental factors. It is also important to bear in mind that the locomotory activity of ladybirds can often be motivated by needs other than the search for food, for example, the search for mates (Honěk, 1985). Although ladybirds in this study were likely motivated to search for prey (given that they had been deprived of aphids, their dietary staple), I cannot know for certain the function of their walking behaviour. On this note, it may also be valuable to explore whether the behaviours seen on artificial and/or horizontal set-ups are more akin to escape behaviour than foraging behaviour. I suspect that if ladybirds are stressed and seeking to escape their situation they are unlikely to behave in the same manner as they would when foraging for prey in an environment which feels natural to them. Perhaps whether escape or foraging behaviour is elicited more on different set-ups could be teased apart by placing aphids on branched set-ups and observing whether ladybirds target them as prey or ignore them. However, the results presented here provide tentative evidence that handedness may not be as important a factor in ladybird search strategies than previous work has suggested. I confirm that ladybirds can indeed show handedness, but may be primarily inclined to do so when other stimuli are not available.

# Chapter 9: The influence of turn alternation and environmental factors on ladybirds searching branched structures

Material from this chapter formed the basis of the publication: Humphreys, R. K., Neuhäuser, M. & Ruxton, G. D. 2021. Turn alternation and the influence of environmental factors on search routes through branched structures by ladybirds (*Coccinella septempunctata* and *Adalia bipunctata*). Behavioural Processes, 181, p.104292.

Turn alternation is a locomotory behaviour wherein an animal makes consecutive turns in opposite directions (left-then-right or right-then-left). It has been suggested that its adaptive function is to maintain locomotion in a relatively constant general direction while negotiating obstacles. Previous work has focussed on the use of turn alternation in prey species in artificial horizontal mazes. In the first study presented here, I tested whether predatory seven-spot ladybirds (*Coccinella septempunctata*) exhibit turn alternation when repeatedly presented with consecutive choice turning decisions on vertically-oriented twigs. The findings suggest that turn alternation occurs vertically as well as horizontally, on plant structures as well as terrestrially, and in a type of animal (predatory insect) in which turn alternation has received little attention. In the second study presented here, I tested whether characteristics of branched structures explored by two-spot ladybirds (*Adalia bipunctata*) influence turning decisions. The findings suggest that ladybirds exhibit preferences for thicker over thinner, straighter over more deviating headings, and higher over lower turning choice options at bifurcations. These exploratory studies indicate that while turn alternation is an observable phenomenon in ladybirds, it is not the only predictor of searching behaviour on branched structures.

## 9.1 Introduction

Turn alternation is the tendency of animals to make a turn in the opposite direction to their most-recent previous turn when travelling through environments. Among vertebrates, rats (Dember and Richman, 1989), cats (Frederickson and Frederickson, 1979), rabbits (Hughes, 1973), fish (Aderman and Dawson, 1970, Fidura and Leberer, 1974, Bögli and Huang, 2017), and primates (including humans) (Vecera et al., 1991, Izumi et al., 2013, Rothacher et al., 2020) all appear to exhibit spontaneous alternation. A diverse range of invertebrates also demonstrate the behaviour, including several species of terrestrial isopods (Watanabe and Iwata, 1956, Kupfermann, 1966, Hughes, 1967), mealworms (Grosslight and Ticknor, 1953), herbivorous bugs (Dingle, 1965), marine crabs (Ramey et al., 2009), fruit flies (Lewis et al., 2017), earthworms (Nakashima et al., 2018), millipedes, and earwigs (Hughes, 1987). Recently, results from a study by O'Shea-Wheller (2019) suggest that honey bees (*Apis mellifera*) tend to turn alternate in branching cavities, where individuals were presented with two consecutive turning decisions between two symmetrical 90 degree branches; however, bees' context-dependent rightward bias in open cavities was the focus of that study. Most investigations into turn alternation have thus far centred on woodlice (Oniscidea) (Watanabe and Iwata, 1956, Kupfermann, 1966, Hughes, 1967, Beale and Webster, 1971, Ono and Takagi, 2006, Hegarty and Kight, 2014, Moriyama et al., 2016), in part due to the fascination surrounding the adaptations that enabled their terrestriality, given their marine ancestry (Broly et al., 2013). Turn alternation is considered to be an important behavioural adaptation contributing to woodlice survival and success on land, though this locomotory activity no doubt provides functional benefits to the many other taxa in which it occurs.

The primary function of turn alternation is suggested to involve maintenance of locomotion in a given general direction (Hughes, 1967), serving as a ‘correcting’ behaviour or ‘compensatory response’ when obstacles necessitate deviations from the original route. For many species, the adaptive function of turn alternation is thought to be efficient movement or escape from less favourable or dangerous environments (Hughes, 1967). Studies into turn alternation in invertebrates typically utilise artificial, horizontally-oriented Y- or T-mazes, presenting subjects with a choice of two paths at a single bifurcation (Czaczkes, 2018). However, while animals such as woodlice do commonly move across flat structures and substrates, many invertebrates also ascend branched plant structures.

The first study I present here pursues several avenues which, to the best of my knowledge, offer novel insights into invertebrate turn alternation. Firstly, subjects were presented with natural bifurcated twig structures on which to make consecutive turning decisions, offering a different substrate to the typical artificial materials of mazes. Secondly, these structures were oriented vertically, rather than horizontally, thereby exploring the potential for turn alternation on plant structures rather than the terrestrial environment. Thirdly, rather than recording a single occurrence of turn alternation or similarity in each individual, this study explored whether individuals show a repeated tendency to show turn alternation. While population-level tendencies to alternate are already implied by current literature, a repeated measures design should more explicitly confirm whether turn alternation is consistently a significant preference of most individuals in a sample; rather than population-level effects being driven only by a few extreme individuals. Finally, this first study investigated the possibility of turn alternation being exhibited by a type of animal (a predatory insect) not extensively studied previously in this context: the seven-spot ladybird *Coccinella septempunctata* L. (Coleoptera: Coccinellidae).

As well as a potential preference for turn alternation over turn repetition, a range of different environmental factors might further influence the turning decisions made by ladybirds when faced with a branching bifurcation, as discussed in Chapter Eight. Using a similar method to the first study, the second study presented here investigated the influence of several different characteristics of natural twigs on the turning decisions of two-spot ladybirds (*Adalia bipunctata*): branch thickness, the difference of the angles presented by each branching option compared to the present heading, and the difference of the angles presented by each branching option compared to the direction of gravity.

## 9.2 Study 1: Turn alternation in ladybirds

### 9.2.1 Materials and methods

15 adult seven-spot ladybirds were collected (without observing any turning choices) along a short stretch alongside the Kinness Burn in St Andrews, UK (LAT: 56.34, LONG: -2.81), during September 2018. Ladybirds were maintained in sterilised, ventilated, clear plastic containers of dimensions 8cm (H) x 15cm (W) x 12cm (D) in groups of no more than 10 individuals, to reduce the chances of cross-infection (as in Chapter Seven), for no more than four days. Handling and maintenance procedures, and environmental conditions were identical to those followed in Chapter Eight.

Seven twigs that offered two approximately symmetrical consecutive choice branching routes, with roughly Y-shaped junctures at all four branching points, were collected from the same area where the subjects were collected. The twigs varied in height, girth, and texture, providing a range of natural variation to test subjects on (Figure 9.1). Each twig had the two branches stemming from the ‘Lower Stem’ (LS) Y-choice juncture randomly designated as ‘Upper Stem Left’ (USL) and ‘Upper Stem Right’ (USR). Following the same left-right perspective, the branches leading from these upper stems were also assigned designated section names (see Figure 9.2 for details). For each trial, one of the seven twigs was affixed to stand upright in the middle of a large, sterilised clear plastic container: 20cm (H) x 32cm (W) x 23cm (D), with the left-right axis of the twig (based on designated sections, Figure 9.2) aligned with the container’s width, as were the vertically-oriented twigs in experiment 1 of Chapter Eight. The first twig a ladybird experienced was drawn randomly from the complete suite of twigs, all with equal probability. After that, consecutive twigs were drawn randomly in the same way from the full suite minus the last twig experienced. Ladybirds could thus experience the same twig more than once but never on consecutive trials.

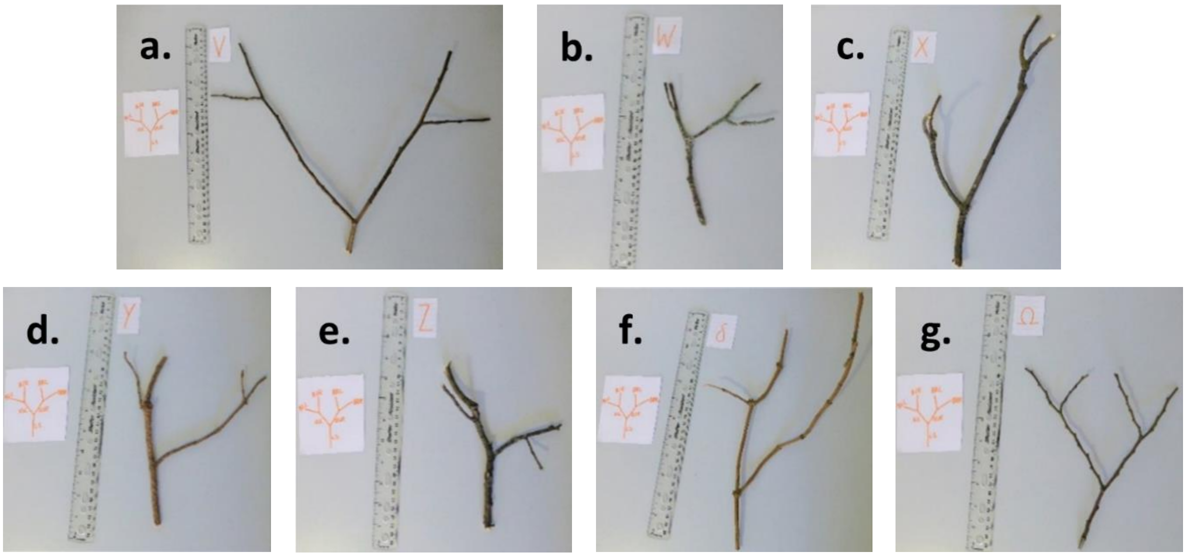


**Figure 9. 1:** The seven twigs with two consecutive choice branching routes (**a-g** corresponding with assigned twig letters V-Z, δ and Ω) each alongside a 30cm ruler. The first-choice right-hand branch of twig δ (**f**) leads to a further choice where one branch continues upwards while another protrudes out towards the camera, making that second choice less clear in this image.

An individual ladybird was randomly selected at the beginning of each 15-trial block (so the 15 ladybirds were evaluated in random order). The ladybird was introduced to the centre of the twig base (max 1.5 cm above the blu tack fixative) from the ‘front’ of the twig (from the left-right perspective, Figure 9.2) using a paintbrush. As ladybirds ascended the twig, it was recorded whether they chose the left or right branch at both the first and second Y-shaped branching points it encountered (using the designated twig sections, Figure 9.2). If a ladybird did not make two consecutive turning decisions (i.e. it back-tracked before reaching either decision point or flew away) the trial was restarted. Between trials with the same subject, the ladybird was placed into a small plastic container while the twig was changed. The apparatus was rotated after each trial to control for any environmental gradients within the room. The repeated set sequence of directions trials were oriented to were as follows, with the angle change between trials noted in parentheses: N (+45°), E (+90°), S (+90°), W (+90°), NE (+135°), SE (+90°), SW (+90°), NW (+90°). During all trials, I remained at a distance, to prevent breath from influencing behaviour, and avoided casting a shadow over the equipment. The room was lit only by natural light. All 15 individual ladybirds completed 15 trials, exhibiting in each either turn alternation (choosing left-then-right branches or vice versa) or turn repetition (choosing two left or two right branches). 225 consecutive choice turning decisions were observed in total.

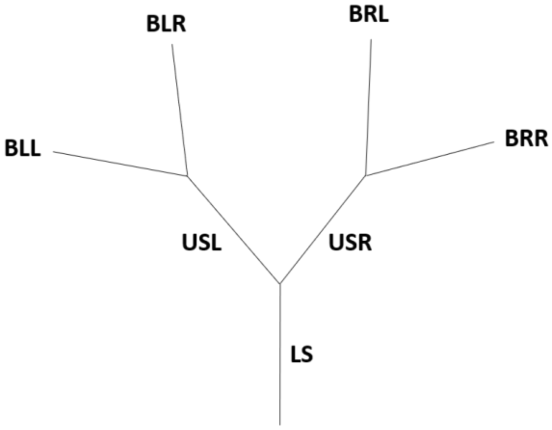


**Figure 9. 2:** The designated sections of twigs, from which turning patterns were determined. The two branches stemming from the 'Lower Stem' (LS) Y-choice juncture were randomly designated as 'Upper Stem Left' (USL) and 'Upper Stem Right' (USR). The branches leading from these upper stems were then labelled according to which left-right choices must be made moving up the stem in order to reach them: BLL = Branch Left Left, BLR = Branch Left Right, BRL = Branch Right Left, and BRR = Branch Right Right. For each twig, these designations were kept constant across trials, so that the assigned 'left' side was always viewed as 'left'.

All analyses were conducted using R version 3.6.3 (R Core Team, 2020) and the observed frequencies of turn alternation and turn repetition exhibited by each individual were analysed using chi-square goodness of fit tests, simulation, and an intercept-only generalised linear model assuming a binomial error with logit-link function.

### 9.2.2 Results

14 of the 15 (93%) individual ladybirds followed turn alternating patterns of consecutive choice more commonly than they followed patterns that involved turn repetition (Figure 9.3). Chi-square goodness of fit tests examined whether the observed turning choice patterns of each individual differed significantly from the null hypothesis that choices were independent and an individual was just as likely to alternate as to make consecutive same-direction choices. The expected cell counts under the null hypothesis were both 7.5. These 15 chi-squared tests demonstrated that in six of the 15 individuals (40%), turn alternation was exhibited to a significant degree: individuals ‘E’, ‘H’, ‘L’ and ‘M’ showed alternation in 12 of their 15 trials (80%) ($\chi^2_1 = 5.4$, $P = 0.02$), while ‘F’ and ‘K’ showed alternation in 13 of their 15 trials (87%) ($\chi^2_1 = 8.1$, $P = 0.005$). No ladybirds exhibited significantly more turn repetition than they did turn alternation.

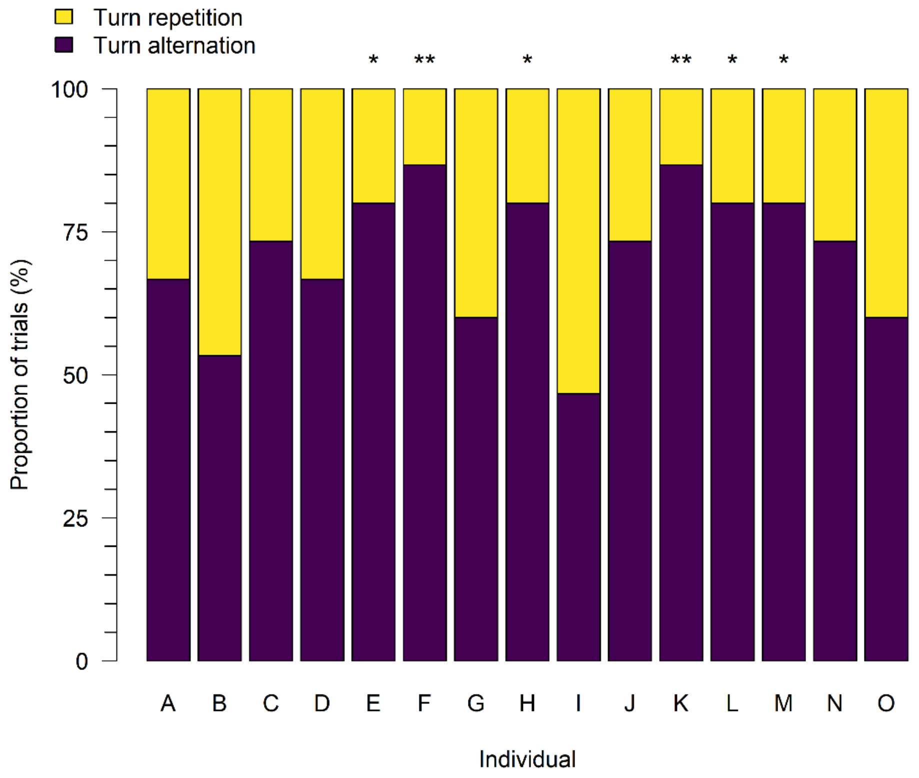


**Figure 9. 3:** The proportion of trials during which each individual seven-spot ladybird (*C. septempunctata*) exhibited turn alternation and turn repetition in the first study. For each of the 15 trials individuals completed, the twig presenting the two consecutive choice branching routes was randomly selected from a range of seven, that varied in height, girth, and texture. 14 of the 15 individual ladybirds exhibited turn alternation more commonly than turn repetition. Chi-square tests found this preference to be statistically significant in six of the 15 individuals; asterisks indicate the resulting p-values, where * < 0.05 and ** < 0.01.

As each individual was evaluated separately in a chi-squared test, it would be expected that a number would test positive for turn alternation in the absence of a true preference simply through type I error. In order to explore this possibility, I conducted a resampling simulation of 10,000 virtual replicates of the experiment, with 15 individuals each completing 15 trials. In these simulations each of the 225 outcomes was independently allocated as alternation or repetition randomly with a 50:50 probability. For each individual, a chi-square goodness of fit test examined whether – by chance alone – the frequency of turn alternation differed significantly from the frequency of turn repetition. None of the 10,000 runs of the experiment produced the outcome that I observed, where six or more individuals exhibited a significant preference for either turning pattern, suggesting that the results of this study cannot be explained by type I error.

Additionally, an intercept-only generalised linear model (GLM) found that the proportion of times ladybirds exhibited turn alternation (outcome variable) was significantly greater (P <0.001) than they exhibited turn repetition (Table 9.1). From the results of this model (Table 9.1), 95% confidence intervals were computed; overall, ladybirds showed turn alternation in 70.8% (±5.9%) of trials.

**Table 9. 1:** GLM output from intercept-only model exploring ladybird propensity to turn alternate. The structure of the model was: glm(propalt~1, family=binomial(link="logit"), weights = trials), where 'propalt' was the proportion of trials in which the ladybird individual alternated, and 'trials' was the 15 trials each individual underwent. The significant p-value is emboldened.

| Estimate | Std. Error | z-value | p-value |
|---|---|---|---|
| 0.9008 | 0.1471 | 6.124 | **<0.001** |

Together, these findings indicate that turn alternation is a behaviour that ladybirds adopt when searching vertically-oriented branched structures.

### 9.2.3 Discussion

This first study provides evidence that turn alternation occurs vertically, on twigs, and is exhibited by predatory insects. Although the number of repeated measures per individual was relatively small, there was a clear tendency across the population to exhibit turn alternation over turn repetition. The randomised use of different twigs and the recording of both right-then-left and left-then-right choices as alternation make it unlikely that the turn alternation observed was simply a result of ladybirds following preferred paths specific to the structures – rather the consecutive choices should be considered as a preference for turn alternation more broadly.

While this study does not in itself reveal why turn alternation may be an adaptive behaviour for ladybirds to adopt when searching branched structures, the functional value may lie in turn alternation being an effective way for individuals to continue heading in a constant general direction (Hughes, 1967). This may be of particular relevance to predatory insects, such as ladybirds, whose prey may often be found on newer leaves and buds towards the periphery of plants (Salyk and Sullivan, 1982, Nakashima and Akashi, 2005, McCornack et al., 2008). Previous experiments with arthropods have suggested that alternating turns can increase the discovery rate of unexploited prey or resource patches in heterogeneous environments (Tuck and Hassall, 2004, Ramey et al., 2009). By allowing for more linear travel up plants and towards leaves, turn alternation may increase a ladybird's chances of encountering prey and decrease energetic and time costs associated with unsuccessful searching. Beyond enabling individuals to continue in a given heading, turn alternation behaviour in ladybirds might also be related to defence mechanisms against injury sources, as seems to influence turning by woodlice (Carbines et al., 1992, Houghtaling and Kight, 2006, Ono and Takagi, 2006, Hegarty and Kight, 2014). Regardless of function, it is also possible that vertical orientation of structures may make turn alternation a more applicable strategy for animals that can sense gravitational variation, as it would presumably be easier to detect deviation from a linear path of movement if such a deviation is also associated with a reduced perceived increase in elevation. That is to say, if a ladybird showed turn repetition when ascending the branches of a plant, the change in elevation may provide an additional cue that correcting turn alternation is subsequently required to resume upward ascent. However, this suggestion certainly requires further study, and may also depend on the mechanism by which turn alternation functions in predatory insects.

It has been suggested that spontaneous alternation in vertebrates is driven primarily by environmental directional and odour cues, while turn alternation in invertebrates is driven primarily by kinaesthetic or tactile cues (Richman et al., 1986). However, some invertebrates have demonstrated alternation governed by environmental cues, such as brightness differences for cockroaches (Wilson and Fowler, 1976) and differences in object sizes and colours for marine crabs (Ramey et al., 2009). Even for terrestrial isopods whose alternation behaviour is assumed to be driven by proprioceptive feedback from legs, some environmental stimuli, tactile cues, and psychological mechanisms, may also play important roles in determining alternation. For example, substrate brightness (Hughes, 1992), substrate-borne vibrations (Cividini and Montesanto, 2018), predator cues (Carbines et al., 1992) and the distance between turning decisions (Hughes, 2008) have all been demonstrated to have some influence on the turning decision made by woodlice. No work I am aware of has explored the influence of forced turns on subsequent turn alternation by ladybirds, but studies into this should prove useful in determining whether proprioceptive cues are critical for driving their turn alternation. Concerning environmental cues, the exploration of turning bias in ladybirds at single turn choices detailed in Chapter Eight found that substrate material and orientation could have significant effects; specifically, no significant patterns of bias were seen on vertically-oriented twigs of either natural or artificial substrate, but when horizontally-oriented significant turning biases were exhibited on artificial but not natural twigs. This indicates that turning preferences are not the only determinants of animal movement through branched environments. As shown previously in woodlice (Carbines et al., 1992, Hughes, 1992), environmental variables – for example, light or gravitational variations, varying substrate textures, or the angle of branching options – may all have some bearing on ladybird turning decisions. As an example, there is evidence that increasing the distance travelled between turning choices, though not increasing the time, decreases the probability of woodlice alternating turns (Hughes, 2008). Whether this pattern occurs in ladybirds, and whether individuals either ‘forget’ the previous turn or effort-related leg fatigue causes a reduced propensity to exhibit turn alternation warrants exploration.

Certainly, any preference for turn alternation will not be the only predictor of ladybird behaviour on plants. I predict that this tendency should be adaptively flexible, as often there may be better cues available to individuals, for example indicators of where foraging may be more successful. Nonetheless, this first study indicates that alternation is an additional, real phenomenon that is not simply a biproduct of decisions relating to other factors, such as the difference of angles at bifurcations relative to an individual’s current heading or the direction of gravity.

## 9.3 Study 2: Influence of twig characteristics

### 9.3.1 Materials and methods

Ten twigs that offered two approximately symmetrical consecutive choice branching routes were collected from Kelvingrove Park and along the last 2km of the River Kelvin in Glasgow, UK (LAT: 55.87, LONG: -4.29), during July 2020. The twigs were sourced from a variety of plant genera (hawthorn *Crataegus* spp., ash *Fraxinus* spp., privet *Ligustrum* spp., oak *Quercus* spp., rhododendron *Rhododendron* spp., willow *Salix* spp., linden *Tilia* spp., elm *Ulmus* spp.) in order to provide ladybirds with a range of different textures and odours. The twigs were similar to those used in the first experiment in that they would present a ladybird walking along them from the starting point with two consecutive choices at bifurcation points. Each section of each twig was designated from a randomly assigned left-right perspective (as in the first study, see Figure 9.2 for ‘upper stem’ and ‘branch’ section labelling as referenced below). The twig section girths, angles relative to previous twig section heading, and angles relative to the direction of gravity were recorded for each different turning choice option at all three bifurcations on each twig. Using these measurements, whether each option resulted in a choice of thicker-or-thinner, straighter-or-more-deviating heading, or higher-or-lower, relative to the alternative option at that bifurcation was determined. 35 adult two-spot ladybirds were supplied by Green Gardener (Rendlesham, Suffolk) and Dragonfli (Halstead, Essex). Ladybirds were maintained in ventilated plastic containers and, as in the first study, no ladybird was kept for more than four days.

Given that this study was designed to investigate environmental variables, to better resemble field conditions trials took place outside in a courtyard and the set-up was positioned so as to experience natural lighting conditions without being shadowed by buildings or fences. For each trial, one of the ten twigs was affixed to stand upright in the middle of a paving stone. For each ladybird, the order of twigs to be tested on was randomised (random.org, 2020), but without replacement between trials, so ladybirds only experienced a given twig once. An individual ladybird was randomly selected at the beginning of each block of trials. The study aimed to test each individual ladybird on all ten twigs in a 10-trial block, but sometimes ladybirds flew away during trials, thus ending their block. The procedure for handling ladybirds, recording turning decisions, and changing the orientation of twigs between trials was the same as followed in the first study.

Twenty five ladybirds completed trials on all ten twigs, and ten further ladybirds completed trials on between one and nine twigs; 284 trials were completed overall. Chi-square goodness of fit tests were used to examine observed frequencies of binary preferences for thicker-or-thinner, straighter-or-more-deviating heading, or higher-or-lower branches at both the first and second choice points (hereafter called the first and second turns) in trials. Generalised linear mixed models (GLMMs) were also fitted to explore these binary preferences, using the *lme4* package (Bates et al., 2015) in R version 3.6.3 (R Core Team, 2020) and assuming a binomial error with logit-link function. For the first and second turn choices, separate random intercept-only GLMMs were constructed to test for difference from 50:50 preference for either of the binary choices (outcome variable) for each characteristic. Models with the first turn choice as a fixed effect (explanatory variable) for the outcome of the second turn choice were also produced, to explore whether the choice at the first turn influenced the choice surrounding each given characteristic at the second turn. For all models, ladybird individual was included as a random intercept, because individuals produced more than one observation in the data.

### 9.3.2 Results

Considering first the potential for a preference between thicker-or-thinner branching options, at the first turning decision the thicker upper stem option was chosen in 187 trials and the thinner upper stem option was chosen in 97 trials ($\chi^2_1 = 28.5$, $P < 0.001$). At the second turning decision, the thicker final branch option was chosen in 180 trials and the thinner final branch option was chosen in 104 trials ($\chi^2_1 = 20.3$, $P < 0.001$). These significant preferences for thicker branches at both turns were also confirmed through GLMM modelling, and the girth choice at the first turn was found to have no significant influence ($P = 0.884$) on the girth choice at the second turn (Table 9.2).

Secondly, it was explored whether ladybirds preferred to maintain a relatively straight heading or to make a greater turn away from their current path to choose a more deviating route. At the first turning decision, the straighter upper stem option was chosen in 192 trials and the more deviating upper stem option was chosen in 92 trials ($\chi^2_1 = 35.2$, $P < 0.001$). At the second turning decision, the straighter final branch option was chosen in 177 trials and the more deviating final branch option was chosen in 107 trials ($\chi^2_1 = 17.3$, $P < 0.001$). Again, these significant preferences were confirmed through GLMM modelling, and the relative heading choice at the first turn was found to have no significant influence (P = 0.486) on the relative heading choice at the second turn (Table 9.2).

**Table 9. 2:** GLMM outputs from models exploring ladybird choices of thicker-or-thinner, straighter-or-more-deviating heading, or higher-or-lower branches at their first and second turns on twigs during the second study. In all models, ladybird individual was included as a random intercept. Significant p-values are emboldened.

| | | **GLMM outputs[1]** | | | |
|---|---|---|---|---|---|
| **Binary choice** | **Model tested[2]** | **Estimate** | **Std. Error** | **z-value** | **p-value** |
| ***Thicker or thinner*** | First turn<br>(random intercept-only model) | -0.656 | 0.125 | -5.246 | **<0.001** |
| | Second turn<br>(random intercept-only model) | -0.552 | 0.128 | -4.314 | **<0.001** |
| | Does the second turn depend on the first turn? | -0.038 | 0.262 | -0.146 | 0.884 |
| ***Deviating or straighter*** | First turn<br>(random intercept-only model) | 0.736 | 0.127 | 5.802 | **<0.001** |
| | Second turn<br>(random intercept-only model) | 0.503 | 0.123 | 4.110 | **<0.001** |
| | Does the second turn depend on the first turn? | -0.184 | 0.264 | -0.696 | 0.486 |
| ***Higher or lower*** | First turn<br>(random intercept-only model) | -0.488 | 0.122 | -3.996 | **<0.001** |
| | Second turn<br>(random intercept-only model) | -0.354 | 0.145 | -2.445 | **0.015** |
| | Does the second turn depend on the first turn? | 0.449 | 0.256 | 1.755 | 0.079 |

[1] given to 3 decimal places

[2] for each model, the first option given in the ‘Binary choice’ column was set as the reference category (alphabetically the first of the two levels per choice)

Finally, it was considered whether ladybirds might have a preference for branches presenting greater or smaller angles compared to the direction of gravity; that is, branching options leading higher or lower relative to the alternative,  At the first turning decision, the higher upper stem option was chosen in 176 trials and the lower upper stem option was chosen in 108 trials ($\chi^2_1 = 16.3$, $P < 0.001$). At the second turning decision, the higher final branch option was chosen in 166 trials and the lower final branch option was chosen in 118 trials ($\chi^2_1 = 8.1$, $P = 0.004$). Both of these preferences for higher branching options were also found to be significant through GLMM modelling. The preference regarding relative angle compared to the direction of gravity taken at the first turn was found to have no significant influence ($P = 0.079$) on the option chosen in terms of relative angle compared to the direction of gravity at the second turn (Table 9.2), though this decision was borderline.

### 9.3.3 Discussion

Overall, this second study provides evidence that some characteristics of branching structures can influence the turning decisions made by predatory insects such as ladybirds. Ladybirds here demonstrated significant tendencies to opt for thicker over thinner, straighter over more deviating, and higher over lower turning choice options. Previously, turning decisions based on environmental factors, such as substrate brightness (Hughes, 1992) and the distance between turning decisions (Hughes, 2008), have been demonstrated in woodlice, but I am not aware of studies exploring factors that influence turning decisions at bifurcations on branched structures.

One potential limitation of this study was that the designations of branching option characteristics (thicker-or-thinner, straighter-or-more-deviating heading, or higher-or-lower) were binary, even though sometimes the different girths or angles between branching options at a given bifurcation were only slight. However, there were no cases where the options were deemed to be indistinguishable in girth or angles, as a quantitative measurement always led to binary designations. Further, there is no reason to assume that ladybirds are incapable of detecting even very slight differences in girth, straightness, or direction relative to gravity, particularly if it is adaptive for them to pursue branching options with particular characteristics relative to the alternative at a bifurcation. However, it is also true that I cannot say confidently that all choices made at bifurcations in this study necessarily resulted from adaptive preferences. Particularly true for some of the thicker-over-thinner and straighter-over-more-deviating turning decisions observed in the study, it would be difficult to demonstrate that these were made due to adaptive decision-making (e.g. perhaps these preferences help ladybirds maintain an upward heading on branched structures, as proposed as a function of turn alternation in the first study), rather than resulting from ladybirds' sensory limitations. In some cases, ladybirds made choices at bifurcations without turning towards, contacting, or showing any awareness of the thinner and/or more deviating heading turning options at all.

## 9.4 Conclusions

The first study presented here suggests that predatory insects, such as ladybirds, exhibit turn alternation when exploring vertically-oriented natural twigs. Alone, this finding does not explain why turn alternation may be an adaptive behaviour for ladybirds searching branched structures, but I propose that such functional value may lie in the maintenance of a constant general direction. The mechanism of turn alternation in ladybirds is also yet to be investigated but, even if the behaviour is driven primarily by proprioceptive cues, some environmental stimuli may also influence turning decisions, as is the case in woodlice (Carbines et al., 1992, Hughes, 1992, Hughes, 2008, Cividini and Montesanto, 2018). Indeed, the second study presented here provides evidence of the influence of several different environmental factors on the turning decisions made by ladybirds: the girth, the straightness of heading, and the angle relative to the direction of gravity, of turning options relative to the alternative option available at bifurcations in twig structures. Ladybirds demonstrated preferences for thicker, straighter, and higher twigs at both first and second turning decisions. Taken together, these exploratory studies indicate that while turn alternation is an observable phenomenon in ladybirds, it is not the only predictor of searching behaviour on branched structures. Future studies could look to explore concurrently how multiple characteristics of a ladybird's environment influence turning decisions, and the influence of any interactions between environmental factors themselves and with the propensity for turn alternation. Such a valuable multivariate analysis was beyond the scope of this paper, but I can imagine a scenario where artificial substrates are utilised to allow the various different factors to be independently manipulated, helping to unpick the key drivers of decision-making by ladybirds at bifurcations.

# Chapter 10: General Discussion

Explorations into how natural selection has shaped the ways in which predators search for prey and how prey, in turn, utilise defensive adaptations to evade predators are central to developing understanding of the ecology, population dynamics, and ultimately evolution of many predator-prey interactions. My focus on behaviours stems from my interest in the relative flexibility and context-sensitivity of behavioural adaptations in comparison with more fixed morphological or physiological traits. In this thesis, I used the ladybird-aphid predator-prey study system to explore the relatively neglected defensive behaviour of dropping (reviewed in Chapter Three) and the movement tendencies of predators searching branched environments. Aphids are important agricultural pests with a near-worldwide distribution (Blackman and Eastop, 2000, van Emden and Harrington, 2007, Dedryver et al., 2010), making their interactions with ladybirds (some of their most common and significant natural enemies) worthy of particular attention. My work on prey dropping covered some of the most obvious internal and external factors that might affect its use, and the possible consequences of subsequent behaviours. My work on predator search involved preliminary observations of search movements on wild plants, but primarily tested for possible turning biases or ‘handedness’ and turn alternation on branched structures.

Viewed alongside the findings of previous studies, the results contained in this thesis indicate that dropping is a significant component of aphids’ antipredator behavioural repertoires, and that it is deployed flexibly and adaptively in order to avoid or reduce associated costs. Considering internal influences on dropping, the results of Chapter Four suggest that the presence of the symbiont *Hamiltonella defensa* alone, irrespective of possible associated parasitism resistance, may affect aphids’ propensity to use different antipredator defences. Future studies should draw comparisons between the behaviours exhibited by aphids infected with different strains of *H. defensa*, offering different levels of parasitism protection, when faced with different predator and parasitoid types, in order to confirm whether symbiont effects on behaviour relate to parasitism resistance or are simply by-products of infection. Clarity on this is important, as it will indicate how adaptive or otherwise the behavioural effects might be during encounters with different types of natural enemy. There is also an urgent need for field studies testing the consequences of reduced defensive behaviours due to *H. defensa* for aphid survival and fitness following natural predator-prey encounters (Sochard et al., 2020). Exploring a range of predator types would also be important in such field studies, as the findings of Chapters Four and Five both suggest that predators with different foraging styles can elicit different behavioural responses in aphids. As detailed in Chapter Five, both pea (*Acyrthosiphon pisum*) and potato (*Macrosiphum euphorbiae*) aphids were found to drop relatively more frequently when faced with ladybird (*Adalia bipunctata*) adults compared to lacewing (*Chrysoperla carnea*) larvae. This supports the suggestion of previous studies that aphids drop more following encounters with larger, more energetically-foraging predators than with smaller, slower-foraging predators (Brown, 1974, Brodsky and Barlow, 1986, Losey and Denno, 1998a, Day et al., 2006, Francke et al., 2008). The study in Chapter Five also identified predator contact and close proximity as significant triggers of aphid dropping, lending support to one of the key arguments of this thesis: that dropping is deployed only when the risk of predation is assessed as being high. This follows because dropping is a costly defence and predators do not always make efficient or targeted attacks. Additional experiments comparing aphid responses to a wider range of predator types with different body sizes, foraging speeds, prey detection abilities, and attack styles – singly and as part of multipredator systems (Brilot et al., 2012) – are required to explore the relevance of such predator traits to aphid threat evaluation and dropping propensity. Ideally, studies should also utilise wild-sourced aphids and natural conditions wherever possible, to best reflect natural behaviours without the potential for responses having been altered by long-term culturing (Thieme and Dixon, 2015).

The results of Chapter Six provide important candidate pathways for future research into post-dropping behaviour, an area that is not commonly considered in studies of antipredator defences. An underappreciated yet immediate post-dropping behaviour, the re-clinging of aphids to the lower-reaches of plants, appeared to depend to some extent on the height of the plants dropped from, but further, more purpose-designed studies quantifying characteristics of plants (e.g. architecture) are needed to capture how commonly this cost-mitigating behaviour occurs and what factors influence its likelihood. The fieldwork described in Chapter Seven also indicated that the likelihood of aphids dropping from plants may depend on the plant understructure present, though further work that quantitatively assesses the understructure of host plants and underlying non-host plants is necessary to confirm this. Also, it should be investigated whether aphids are able to detect or assess the density of the understructure below, but I suspect that any link between dropping likelihood and the understructure present below may be more of a result of the coevolutionary histories between different aphid species and their most common host plants rather than in-the-moment individual assessments of re-clinging likelihood. Further, as discussed in Chapter Seven, host plant growth form has likely played a significant role in shaping the dropping behaviour of leaf beetles over evolutionary time (Matsubara and Sugiura, 2021), a suggestion that may also hold true for aphids. However, testing of these conjectures would be very much worthwhile.

Turning to the majority of cases in Chapter Six, where aphids dropped all the way to the underlying substrate, my work suggests that external factors such as environmental conditions and the stimulus for dropping can significantly influence aphids' post-dropping tonic immobility (TI) and rate of recovery back to feeding sites. Certainly, more studies investigating the role of dropping stimulus in determining aphids' subsequent recovery locomotion are necessary in order to more fully understand the impact of dropping on aphid fitness, both on individual and population scales. Dropped aphids have been reported as adopting different characteristic movements, often travelling greater distances after dropping in response to a predator threat and travelling shorter distances while turning more after dropping in response to mechanical touch (Niku, 1975, Phelan et al., 1976, Wohlers, 1981). Ben-Ari et al. (2015) also demonstrated that aphids possess surprising ground movement capabilities, and are able to walk many metres across the ground before ascending plants. Further testing is required in order to determine whether the great distances some aphids travelled after dropping in that study are aberrant or adaptive. That is, are they a consequence of confusion, dehydration, or an 'over-reaction', or are they a fitness-enhancing behaviour that gets them clear of an area where predators are present? Alongside controlling for environmental conditions, any studies exploring this question should of course bear in mind that the 'adaptive' view of the behaviour is dependent on aphids being unlikely to travel to an area where there are also foliar-foraging predators present, or significantly increasing their exposure to ground-foraging predators during their recovery journey.

TI, although studied as an antipredator behaviour in itself across a wide range of taxa (see Ruxton et al. [2018] and references therein), is a further avenue from Chapter Six that will likely yield interesting findings when considered more in light of dropping behaviour. Unlike many other cases of TI studied so far where the display of TI is targeted at the threat that directly triggered an antipredator defence, when aphids drop from plants in response to a predator the TI they exhibit would presumably not function to help them evade the predator that they have just fallen some distance away from. Instead, TI once on the substrate presumably serves to help aphids reduce localisation by predators on the ground, despite the fact that there may be none immediately present. It might be argued that post-drop TI helps aphids evade visual, avian predators that might have observed the motion of the individual dropping from a plant, but it seems less likely to me that birds are more commonly a threat to dropped aphids than ground predators, particularly in areas with a high density of plants. It was beyond the scope of this thesis to explore the adaptive value of post-drop TI, but it would be of great interest if future studies could explore whether it is effective at reducing localisation by ground predators that may have detected the dropping motion. As part of this, I predict that the cues and mechanisms for ending a state of TI might be different where it occurs following a drop compared to when it is displayed immediately in the same location as the predatory threat. In the latter case, for example, the displaying individual might be able to pick up on visual and vibrational cues to assess whether the predatory threat remains interested in them as prey or whether it is safe to resume normal activity. By contrast, it would likely be harder for dropped individuals to immediately discern whether any ground-based predators are present or how likely it is that their fall was detected, and so more difficult to determine whether it is safe to start moving again without being tracked. This may be especially challenging where there is a diverse ground-based predator community that could possibly attack. Studies with beetles suggest that vibrations of particular strength can arouse individuals from states of TI, and that sensitivity to arousal from TI could have a genetic basis (Miyatake et al., 2019), but I am not aware of any investigations into the factors that might prompt aphids to exit states of TI.

The threat posed to aphids upon exiting a state of TI would, of course, depend on the senses utilised by their predators in prey detection. As a speculative suggestion, if an aphid were to drop from a crop plant in a monoculture field wherein it has fed exclusively on that crop, perhaps the aphid would benefit from non-visual, olfactory crypsis (Ruxton, 2009) to some extent due to its diet. In such a case, olfactory detection at short range may be especially tricky for predators, and so if they strongly relied on visual cues then TI might be an effective way of evading detection if predators’ attention is caught by the drop itself. This is highly speculative, though, and the adaptive value of post-drop TI will only successfully be identified through a series of purpose-designed studies using multiple predator types, ideally with set-ups that reflect natural conditions as much as possible.

In general, the findings of Chapter Six indicated that what happens to aphids after dropping could have significant consequences for fitness on individual and population scales, particularly if dropping occurs frequently across repeated encounters with predators, but within this PhD there was not time nor resources to explicitly explore the longer-term costs of dropping. As discussed in Chapter Three, dropping has been found to be an important non-consumptive effect of predators, because it can reduce or delay reproductive output and thereby slow population growth (Nelson et al., 2004). Nelson (2007) simulated regular disturbance of pea aphid nymphs by removing them from their host plants for various durations, finding that aphids disturbed for longer periods of time suffered considerable reproductive costs. This suggests that aphids are unable to compensate for lost feeding time by adjusting their activity budgets alone, an issue potentially arising from the fact that their food source is nutrient-poor (Nelson, 2007), as discussed in Chapter Two and the Appendix. More recently, Agabiti et al. (2016) also investigated the impacts of disturbance on pea aphid nymphs, though dropping by aphids was elicited by gently tapping plants and their disturbance treatments varied in frequency rather than duration. Increased dropping behaviour had a strong negative effect on the development of nymphs and their subsequent reproductive capacity as adults. Interestingly, the time taken for nymphs to return to plants by re-climbing grew progressively longer across the experiment for aphids in the high disturbance treatment group (Agabiti et al., 2016). In Chapter Six, I found that return rates of potato aphids post-dropping were significantly affected by air temperature and the stimulus that triggered the drop, but experiments exploring the influence of different factors on recovery rates that can track repeated disturbance of individuals might reveal important longer-term interactions and changing relationships between environmental factors and recovery rates over time. Consideration of how the time aphids spend exposed to abiotic challenges and ground-active predators while recovering will also feed into how dropping could further suppress population development (Agabiti et al., 2016). The impact of disturbance on subsequent fitness will also likely depend on the life stage at which aphids are regularly induced to drop. Further studies into the impact of dropping on the fecundity of older instars and adults might also be valuable, although recovery back to plants may involve less of an energetic expense to bigger, more mature individuals. Additionally, due to the ‘telescoping’ of generations seen within aphid parthenogenetic reproduction (Dixon, 2012 - discussed more in the Appendix), mature aphids may already be carrying well-developed embryos and so their reproductive output should not be reduced by the disruption of dropping. It would be interesting to explore, though, whether increased dropping by grand-maternal or great-grand-maternal individuals subsequently affects the development and fitness of the offspring resulting from the still-developing embryos within the already-developed embryos carried by those individuals experiencing a high frequency of disturbance.

In order to contribute usefully to pest suppression management practices, efforts should also be made to quantify the disturbance experienced by aphids encountering natural enemies throughout their lifecycles, and to what extent this impacts fecundity (Culshaw-Maurer et al., 2020). The ratio of non-consumptive defence-inducing encounters with predators (‘disruptions’) and consumptive encounters for different aphid species has been suggested by Nelson and Rosenheim (2006) as being a useful indicator of the mechanism of pest suppression by predators. Where disturbed:consumed ratios are low, predator-prey dynamics are dominated by the consumption of prey, but where disturbed:consumed ratios are high, predator-prey dynamics have the potential to be governed by dropping, leading to subsequent reductions in reproductive output. Such ratios can be highly species-dependent, in terms of both the predator and prey involved as, for example, Nelson and Rosenheim (2006) demonstrated that the potential for predator suppression of pea aphid populations through induction of defensive behaviours is high, but low for cotton aphid (*Aphis gossypii*) populations (as this species does not typically express escape responses like dropping to predators). Although limited to situations where disturbance results in costly behavioural responses and limited by the fact that ratios are unlikely to be fixed for any predator-prey system, such a measure could still usefully contribute to understanding of how predator-induced disturbances help to suppress prey populations (Nelson and Rosenheim, 2006).

A further major topic for the field of dropping that was beyond the scope of this PhD concerns the fitness consequences of dropping and its influence on a population or species over evolutionary time. The differential use of dropping behaviour reported in Chapters Four and Five suggests that this defence represents a trade-off between various advantages and disadvantages to individuals under different circumstances; for the species tested in this thesis, adaptive decision-making appears to be involved in its use. Further, the results of Chapter Six indicate that the behaviours expressed after dropping also vary depending on a range of factors, potentially increasing the time prey insects such as aphids spend off-plant and exposed to associated risks. It is fair to say that, despite a limited number of studies suggesting that dropping may increase nymphal development time and reduce lifetime fecundity in aphids (Roitberg et al., 1979, Nelson, 2007, Agabiti et al., 2016), not much is known yet about the fitness consequences of dropping, particularly in the long-term. Crucially, as far as I am aware, no study has yet directly demonstrated that propensity to drop is heritable – in aphids or, indeed, other species – but I think there is too much circumstantial evidence for it to not be a heritable adaptation. The variation in the use of dropping seen between some aphid species (Montgomery and Nault, 1977, Hajek and Dahlsten, 1987, Losey and Denno, 1998b, Nelson and Rosenheim, 2006), for example, indicates that natural selection shapes this trait and that it is linked to the genetic dissimilarities that arise between reproductively-isolated species. The fact that dropping has further been found to vary between aphid races (Kunert et al., 2010), clones (Braendle and Weisser, 2001, Stacey and Fellowes, 2002) and biotypes (Ben-Ari et al., 2019) adds support to the idea that this is a phenotype associated with genetic divergence on some level and not environmental context alone. For example, Ben-Ari et al. (2019) found that pea aphid biotypes – that is, genetically distinct populations adapted to feeding on different host legumes – showed great variability in their dropping response to simulated mammalian breath. However, within-biotype variability in dropping tendency was low and the lack of relationships found between the inter-biotype behavioural differences and their genetic distances from each other provide strong support that the variability is adaptive and heritable (Ben-Ari et al., 2019). Whether behavioural divergences result primarily from differences in the selective pressures exerted on different races, clones, or biotypes, or from different lengths of evolutionary history spent genetically isolated (Kunert et al., 2010, Ben-Ari et al., 2019), the adaptive explanation for the divergences is not always clear. But more comparative studies exploring the heritability of the behaviour through different populations experiencing different conditions should shed some light on this.

Particularly interesting in the case of aphids, clonal aphids are often found to exhibit intra-clonal behavioural differences. Despite aphids commonly reproducing parthenogenetically, as discussed in Chapter Two and the Appendix, individuals appear to maintain genotypic and phenotypic variability. This was explored in a study by Andrade and Roitberg (1995), which found that although offspring of a single parthenogenetic female often consist of a mixture of dropping and non-dropping phenotypes (Roitberg and Myers, 1978, 1979), artificial selection led to significant changes in the proportion of clonal pea aphids expressing these two phenotypes. This supports the suggestion that variation in pea aphid dropping behaviour may have a heritable basis. Because of the ‘telescoping’ of generations seen within aphid parthenogenetic reproduction (Dixon, 2012 - discussed more in the Appendix), aphid phenotypes can be influenced by environmental effects on the maternal or grand-maternal generation, and it is possible that such effects also influenced the response of clones to selection in the study by Andrade and Roitberg (1995). But, as highlighted by the authors, future studies that can make genetic comparisons of dropping and non-dropping individuals and rear sexual morphs would provide useful insight as to how far dropping propensity is genetically-determined, how heritable it is, and how repeatable it is within individuals.

Certainly, dropping would not be the first antipredator behaviour that appears to be heritable in insects. Studies into TI have utilised artificially selected lines of various beetle species, bred for either shorter or longer durations of post-contact TI, finding evidence that the trait can be inherited (Miyatake et al., 2004, Ohno and Miyatake, 2007). Recently, Uchiyama et al. (2019) found over 500 differentially expressed genes between red flour beetle (*Tribolium castaneum*) strains bred to exhibit long- or short-duration TI. In these beetles, strains selected for longer duration TI have been found to have a fitness advantage over those selected for shorter duration TI, in that they experience a reduced frequency of predation when faced with a specific predator (Miyatake et al., 2004). However, the fitness consequences of a behavioural tendency are not always clear. In the case of dropping by aphids, it would be of great interest if lines of dropping and non-dropping individuals could be bred and have their fitness quantified in semi-natural conditions. But this would be complicated by the fact that dropping is just one option in a suite of possible antipredator defences, as discussed in Chapters Four and Five. That is, a dropping population may not necessarily outperform a non-dropping population when facing a predator because the non-dropping population might perform highly-effective kicking or running behaviours, for example, to defend themselves instead.

Once dropping genes have been identified, it will also be important for researchers to investigate what the behaviour might correlate with genetically and what genetic trade-offs might be involved. In a study of TI in the adzuki bean beetle (*Callosobruchus chinensis*), for example, longer TI durations were reported as positively correlating with a range of fitness-enhancing life-history traits, including longevity, emergence rates, egg size, development rate, and reproductive effort (Nakayama and Miyatake, 2008). However, in the red flour beetle, a strain selected for longer durations of TI were found to be significantly more sensitive to environmental stressors, including high or low temperatures and mechanical vibrations (Kiyotake et al., 2014). There also seems to be a genetic trade-off in this species between individuals' ability to mate and to avoid predation, as strains exhibiting longer durations of TI had higher survival rates in predatory environments but lower activity and mating success, even in predator-free environments (Nakayama and Miyatake, 2010b). In the adzuki bean beetle there has been found a similar negative genetic correlation between TI and locomotor activity, wherein predator avoidance seems to be traded-off with mating success, though this was only the case for males not females (Nakayama and Miyatake, 2010a). Whether any behavioural and genetic correlations found to be associated with dropping in any species are reflected in the same way in both sexes should also be carefully considered, as this would have significant consequences for the ecology and population dynamics of a species.

It must be acknowledged here that not all studies support the idea of dropping propensity being a heritable trait. The artificial selection employed by some studies does not always succeed in influencing the frequency of dropping response in subsequent generations, and patterns of clonal variation in dropping behaviour are not always consistent (Clegg and Barlow, 1982, Brodsky and Barlow, 1986, Braendle and Weisser, 2001). These studies have concluded that, rather than antipredator behaviours being heritable, the behaviours aphids exhibit depend primarily on the type and strength of the dropping stimulus (Clegg and Barlow, 1982, Brodsky and Barlow, 1986). However, it is clear from other works that genetic differences between aphid populations in their propensity to use dropping do exist (Roitberg and Myers, 1978, Roitberg and Myers, 1979, Dill et al., 1990, Andrade and Roitberg, 1995, Ben-Ari et al., 2019). Certainly, as I found in Chapters Four and Five, external factors such as predator type can have a significant impact on the use of dropping by aphids, but that is not to say that dropping likelihood is not an inherited trait. Even if a disposition to drop is inherited, this does not mean that it is fixed so rigidly that an individual or all individuals within a clone will consistently use only dropping as a defence when provoked. Variability exists in the expression of many inherited behaviours, within and between individuals, and so a lack of repeatability does not automatically rule out heritability. Schuett et al. (2011) have also suggested that current understanding of the extent to which behaviours are genetically fixed may be complicated by individual 'personality'. Their study found that genetically identical clonal pea aphids expressed various phenotypes in response to predator attack so that behaviour was not repeatable at the clonal level, but individuals showed repeatability of their responses both within a day and over the course of five days. Different clones, though, differed in their tendency to drop and were found to produce different proportions of the phenotypes explored (consistent dropper, consistent non-dropper, and inconsistent), with some clones not producing all three phenotypes. While the functional explanation of consistent behavioural differences between individuals remains untested, Schuett et al. (2011) suggest that there might be conditions under which behavioural consistency is favoured and other conditions under which flexibility is favoured. Propensity to drop in aphids, then, does not seem to be genetically fixed per se, but response tendencies might be genetically determined and environmental factors (including maternal effects) may shape the development of personality variation at the individual level (Schuett et al., 2011). Further work by Schuett et al. (2015) has since found that life-history trade-offs likely mediate the personality variation found in aphids, but that the effects could also vary depending on interactions with other characteristics (such as colour morph, in their study). Moving forward, I think that experiments designed to test the heritability and expression of dropping phenotypes should strive to utilise more natural set-ups, ideally with free-roaming predators, and test responses over many generations and under a variety of conditions. These would pose practical challenges, and may require specialist equipment that can track individual insects, but there is a need for research to unpack – as far as possible – the extent to which dropping depends on genetics, maternal effects, environmental conditions, and individual state. Such information could teach us a lot about the evolution of dropping as an antipredator defence, why it is so prevalent in some insect species but not in others, and what its long-term effects on aphid populations might mean for pest control in agricultural settings.

As part of the research reported in this thesis, I attempted to collect some very preliminary data on predator-prey interactions on wild plants under natural conditions. Chapter Seven details my exploratory field study aiming to stimulate further research into both sides of the ladybird-aphid system: predator searching behaviour and prey antipredator defences. During this study, antipredator dropping by aphids was only rarely observed, supporting my suggestion from previous chapters that dropping may only be worth the associated costs for individuals when the risk of predation is assessed as being high. Further, the ladybird predators were not seen to search wild plants efficiently, often making turning decisions away from prey locations and rarely capturing prey even after making contact with them. This lends additional support to my suggestion in Chapter Five that dropping should only occur after contact because predators may not commonly detect or target aphids and that first contact from a predator does not typically result in complete subjugation. Aphids, and other insect taxa, should benefit from using dropping cautiously in order to avoid its potentially substantial costs (Roitberg and Myers, 1979, Dill et al., 1990, Losey and Denno, 1998a, Nelson et al., 2004, Nelson and Rosenheim, 2006, Nelson, 2007, Agabiti et al., 2016), only resorting to it when predation seems imminent. The precise costs of dropping will naturally vary between species. Matsubara and Sugiura (2021) recently highlighted the need to test the relevance of diet and body colouration on the evolution of dropping behaviour in phytophagous insects, as generalist feeders are more likely to find suitable host plants post-dropping than specialist feeders, and species with cryptic body colour would be less easily detected by predators post-dropping compared to those with aposematic body colour, for example. Phylogenetic analyses of aphid species combined with ecological and behavioural studies may well prove insightful.

Returning to the idea of threat evaluation by prey, future studies testing specific hypotheses under more controlled conditions will be valuable in revealing more specific conditions under which dropping is triggered. For example, I think that quantitative studies into predator approach style and trajectory relative to the orientation of prey might uncover important insights into prey individuals’ threat evaluation and subsequent use of different antipredator defences. Some aphid species are less likely to drop and more likely to walk away from predators when approached from the front rather than from the rear (Dixon, 1958, Hajek and Dahlsten, 1987), which makes sense given that a frontal approach would carry a strong visual cue of threat (Gish, 2021). Any similarities or difference found across aphid species or prey taxa beyond aphids in this respect would also likely depend on the relative importance of different senses they use to detect approaching threats and their co-evolutionary histories with the natural enemies they encounter most frequently. Whether insect species can differentiate between predators that are actively hunting and those that are satiated or engaging in non-foraging activity when moving through environments would also be of significance to their threat evaluation; as discussed in Chapter Seven, predator inspection by invertebrates has only recently been the focus of some studies (Zaguri and Hawlena, 2019), and more work on insects’ abilities to assess predator state and the risk of imminent attack will certainly be of great interest. Beyond dropping alone, in this field study I observed lots of aphids running quickly away from plant sections with ladybirds present. Due to my focus in this thesis, I was unable to closely examine what became of these aphids, but it made me question: how far are aphids likely to run after escaping a section, and what factors affect where and when they are likely to re-settle? More wild studies are needed to understand the bigger picture of predator-prey interactions on living plants.

Chapters Eight and Nine of this thesis focussed on the predator side of predator-prey interactions on plants by examining the search tendencies of ladybirds (*Coccinella septempunctata* and *Adalia bipunctata*). My study in Chapter Eight found that the orientation and material of branched structures could influence ladybirds' tendency to exhibit a turning bias (or 'handedness'), a theoretically efficient searching strategy (Girling et al., 2007). However, its findings suggest that in natural environments these predators are unlikely to exhibit consistent turning biases and I propose that ladybirds may not have been strongly selected to search plants in the most efficient way possible, given that they are highly chemically defended and therefore at low risk of predation while foraging. This ties in with the finding from the field work in Chapter Seven that ladybirds did not often make turning decisions that led them to prey, and with previous research that suggests ladybird larvae are inefficient at searching plants, frequently wasting time and re-visiting areas without prey present (Banks, 1957). If ladybirds do follow some sort of searching strategy when foraging for prey, I suggest that it may be far more flexible than an innate turning tendency and instead depend primarily on variable environmental factors. The findings of Chapter Nine support this idea as I found that, when faced with consecutive turning choices on branched structures, ladybirds exhibit turning preferences for thicker over thinner, straighter over more deviating, and higher over lower turning choice options; such tendencies will often correlate with changes in gravitational or light cues. The results of Chapter Nine also indicated that ladybirds do exhibit a tendency to alternate turns when making consecutive turning choices. Over the course of evolutionary history, it is certainly likely that ladybirds have developed some innate tendencies when foraging, and turn alternation could be an effective means to keep individuals moving in a relatively linear direction (Hughes, 1967) up plants, for example. However, I predict that this will be an adaptively flexible searching behaviour that could be repressed when faced with more valuable cues about where might be more fruitful locations in which to search for prey. In order to test this prediction, though, a much closer examination of the environmental factors that might influence turning decisions and the potential interactions between these and any propensity for turn alternation is required. I hope that the work in this thesis may inspire such a multivariate analysis, perhaps as part of a controlled study where artificial structures and conditions can be independently manipulated. More generally, understanding of ladybird searching behaviour – and indeed the searching behaviour of all predators on plants – will be greatly advanced by work that tracks foraging for prey on real branched plants while monitoring differences in predator capture rates and prey antipredator escape behaviours alongside variations in the traits of the plants and broader environmental factors.

Overall, this thesis presents novel findings and potentially valuable avenues for future research concerning the use of prey dropping and predator searching behaviour by insects on plants and branched structures. Both aspects of this thesis are important in understanding the temporal and spatial dynamics, outcomes, and consequences of predator-prey interactions. Expanding knowledge in this field, particularly with regards to agricultural pest species, is of increasing importance in a world where the global population is expected to increase further over coming decades (Population Reference Bureau, 2021). Although recent modelling predicts that the global population may decline after a peak within this century, the future trajectories of demographics vary dramatically between countries and it is expected that by 2100 many countries – particularly in sub-Saharan Africa – will witness up to an eight-times increase in their current population sizes (Ezeh et al., 2020, Vollset et al., 2020). Naturally, this raises important issues of food security and, given that the ecological factors that impinge on agriculture are concurrently changing at an unprecedented rate (Wheeler and von Braun, 2013, Morison and Matthews, 2016), any research that could contribute to improvements in the efficiency or sustainability of agricultural practices is urgently needed (Alexandratos and Bruinsma, 2012, Pawlak and Kołodziejczak, 2020).

Pests represent a major threat to agriculture, with arthropod species accounting for around 20% of crop losses on a global scale (equating to over US$ 470 billion) and far higher losses in many developing countries where population growth is predicted to outpace food production (Culliney, 2014, Fernandez et al., 2022). Despite increases in the use of pesticides over recent decades (Oerke, 2006), a major threat to pests remains the risk of consumption by predators. As touched on in Chapter One, predation is a huge selective force for prey species throughout the natural world and, as a consequence, a wide variety of antipredator defence adaptations can be found across taxa. Dropping is one such antipredator defence, but one that has been greatly neglected, likely due to a lack of appreciation of it as a voluntary behaviour and due to its lack of associated morphological or physiological features (discussed previously in Chapter Three). These very factors that make dropping easy to overlook are precisely what makes it likely to be a widespread behaviour, including in many agricultural pest species.

Dropping is certainly a conceptually simple defence, but my thesis contributes to understanding that the factors that influence its use are multifarious and highly context-dependent. Unfortunately for researchers aspiring to elicit dropping behaviour as part of pest management strategies, there will not be any single, simple rule for its use by a given pest individual or species. The influencers and outcomes of dropping will vary on a case-by-case basis, differing between individuals, populations, species, and environmental circumstances. However, this is not to say that we have no means by which to exploit furthered understanding of dropping. For example, in my experiments (detailed in Chapters Four and Five), ladybird adults triggered dropping by aphids far more frequently than lacewing larvae, and if dropping is found to decrease the fitness of dropped pests then this finding could inform natural enemy selection for biological control. A recent study by Alhadidi et al. (2019) found that combined application of various roaming coccinellid predators and parasitoids did not consistently improve biocontrol of pea aphids. But if, as my work provides further evidence for, coccinellids are likely to trigger dropping, then combined use of ladybirds with ground-foraging predators that are perhaps better able to take advantage of dropped individuals might be a more effective combination of functional types for biocontrol. Although combining natural enemy types can sometimes have negative implications for aphid control – through interference or antagonistic interactions (Meisner et al., 2011, Wang et al., 2021) – a synergistic effect of foliar-foraging and ground-based predators on pest suppression, mediated by dropping, has previously been reported by several studies (Winder, 1990, Winder et al., 1994, Grez et al., 2011, Winder et al., 2012) and seems a valuable interaction for pest management strategies to consider.

Pest management strategies might also be optimised by factoring in greater understanding of what happens to aphid pests after dropping. An interesting aspect of dropping highlighted by this thesis is the potential for aphids to re-cling to lower plant parts, thus reducing the behaviour’s cost to them in terms of time and energy. It may be valuable for future research to explore whether certain breeds of crop could be selected or genetically modified such that their morphology makes re-clinging less of a possibility for pest species that would suffer greatly from regularly dropping all the way to the ground. For instance, aphid species that typically settle on the upper reaches of plants and for whom dropping all the way to the ground rather than to lower plant parts would require a lot of energy to be expended on re-ascending. Or perhaps populations in an environment where it is likely they will encounter ground-based natural enemies; such enemies could be present naturally or introduced as part of an integrated pest management strategy. For aphids that do drop all the way to the ground, location of suitable host plants could then also be made more challenging through the practice of intercropping. My thesis found that potato aphids' post-drop recovery rate was influenced by drop-triggering stimulus type and temperature, but another major influence on successful recovery to host plants will be the physical environment and the plant types near to the original host plant. A study by Xu et al. (2017) found that, in the presence of ladybirds, more bird cherry-oat aphids (*Rhopalosiphum padi*) are found on plants in monoculture than they are in a set-up with intercropping. The authors suggest that this may be a result of the physical barriers and non-host chemical cues from the different plant types confusing and hindering the aphids' host plant location abilities, and I think that future work may find that intercropping practices may usefully disrupt the dispersal and fitness of many pest species that use antipredator dropping.

More generally, the findings of my thesis suggest that dropping is an important non-consumptive effect of predators for aphid species, and that pest management strategies may make more informed and effective use of resources through greater appreciation of this behaviour. Incorporating understanding of, and further work on, dropping into predictive models about the effect of predators on prey movements, survival, and reproductive fitness will help build a fuller picture of the quantities, combinations, and types of natural enemy that should be promoted in, or introduced to, crop systems for the most effective biocontrol. Similarly, testing which insecticides might cause pest species to drop could lend valuable insights into their likely effects on pest population growth and/or transmission of plant diseases (Agabiti et al., 2016). Certain insecticides, such as demeton-S-methyl (Dixon and McKinlay, 1992), are known to cause aphids to fall from plants, but further work is needed to determine whether treated aphids make a voluntary decision to leave plants or impaired neurotransmission and muscle function resulting from the treatment causes them to lose their grip.

Beyond aphids alone, increased awareness of dropping may lead to the discovery of new methods to tackle other arthropod pest species that cause substantial damage to agricultural yields. For example, larvae of the diamondback moth (*Plutella xylostella*) are hugely destructive pests of brassicas, and recent work suggests that climate change may in fact be promoting pesticide resistance in this species by expanding its overwintering range and thereby facilitating local persistence all year round (Ma et al., 2021). As discussed in Chapter Three, lepidopteran larvae can drop from feeding sites when disturbed by the presence of a predator (Castellanos and Barbosa, 2006), with some using silk 'drop-lines' to either drop further or re-ascend once the threat has passed (Fitzpatrick et al., 1994, Sugiura and Yamazaki, 2006, Johnson et al., 2007). The consequences of larval dropping on individual development and fecundity, and subsequent impact on population dynamics, has not to my knowledge been explored for lepidopterans. A study by Wang and Keller (2002) found that parasitoids of diamondback moth larvae can employ very different host-attacking strategies when their prey drop from silk line. The specialist parasitoid species *Diadegma semiclausum* typically waits near the silk thread for a suspended larvae to reascend back to the leaf before attacking, while the more generalist parasitoid *Cotesia plutellae* would usually pursue its host down the silk strand and drop onto the ground if the larvae had dropped away (Wang and Keller, 2002). However, I am not aware of further studies testing the variability of dropping by diamondback moth larvae, and whether this differs depending on natural enemy-specific cues. As I found to be the case with aphids, I predict that internal factors relating to the larvae itself, external factors relating to its natural enemies, and environmental factors relating to its host plant and the conditions of its immediate surroundings are likely to feed into an individual's propensity to drop and the consequences of any given drop for its fitness. Closer examination of the genetics linked to dropping behaviour may also offer important insights for control of pest species populations, and this might be more feasible for insects that have already had their entire genomes sequenced. Although this is true for only relatively few crop pests, those sequenced include the pea aphid, the Russian wheat aphid (*Diuraphis noxia*), the diamondback moth (*P. xylostella*), the Asiatic rice borer moth (*Chilo suppressalis*), the cotton bollworm moth (*Helicoverpa armigera*) and the corn earworm moth (*Helicoverpa zea*) (Li et al., 2019).

It must also be noted that the dropping behaviour of pest species is not the only case of dropping that could affect the efficacy of biocontrol. Various studies have reported intraguild attacks by lacewing and ladybird adults triggering defensive dropping by ladybird larvae (Lucas et al., 1997, Sato et al., 2005). For early instars of some ladybirds, dropping can represent an important component of their defensive strategy, helping to reduce the mortality caused by other predators. I think it would be interesting to explore if, like some of the aphids

I observed in Chapters 6 and 7, ladybird larvae are capable of re-clinging to lower plant parts so as to reduce the energy costs of dropping. I am not aware of any studies into whether they could adopt an aerial-righting posture like that of pea aphids (Ribak et al., 2013, Meresman et al., 2014), but such righting is seen in various other arthropods (Jusufi et al., 2011, Zeng et al., 2017). However, young ladybirds of different species seem to use dropping to differing extents (Sato et al., 2005). Further research into this variation could help inform effective combinations of predators that can be usefully introduced alongside one another as biological control agents, and help to explain the relative success of different native ladybird species in light of the threat of intraguild predation from invasive species.

Finally, practical application of knowledge of dropping will further be made possible by increased appreciation of this thesis' finding that the usage and consequences of dropping will be affected by where on a plant a predator-prey interaction occurs and whether a predator succeeds in contacting prey. Most interactions in the experimental work outlined in Chapters Four, Five, Six, and Seven occurred on the undersides of leaves, and the majority of cases where aphids dropped in Chapters Five and Seven occurred after contact with a predator. Whether a predator is able to successfully encounter prey and where on a plant such encounters are likely to occur will be greatly influenced by the search strategies of predators. The searching behaviour of natural enemies is an aspect of predator-prey interactions that has been greatly understudied, particularly in light of its importance in relation to directional and highly context-dependent defensive behaviours such as dropping. The movements of predators, and therefore where and when interactions occur in environments, will have a major influence of whether dropping occurs and what the consequences of dropping will be.

My work indicates that ladybirds may possess innate turning tendencies, but also a sensitivity to environmental cues and conditions that is likely to guide searching movements and potentially override default turning decisions. With further work, such findings could be used to help shape a number of components in agricultural systems in order to maximise predators' effectiveness at pest suppression. For example, as discussed earlier in relation to minimising the fitness advantage dropping provides to pests, it is possible that breeds of crops could be selected or genetically modified for the purposes of pest control. In this case, modifications to the morphology could make travel towards the sections of plants where pests are likely to settle simpler for predators – perhaps through exploiting predator preferences for certain characteristics at branching points, or generally making the texture of plants easier to move around on. In a similar vein, depending on the findings of future studies, there might be clear changes that could be made to tillage regimes, or the plants that are allowed to grow in the understory of crops, in order to facilitate greater ease of movement for predators through crop systems and increase the frequency of predator encounters with pest species. There might also be potential for the use of artificial lighting equipment around crop field settings on cloudier days if, for example, light is found to be a major cue to optimise predator searching on plants. However, some ladybirds are thought to be highly effective nocturnal foragers through the following of olfactory cues (Norkute et al., 2020), so perhaps expensive lighting would never be a cost-effective tactic for pest management.

There is great scope for future research into the potential for both pest species dropping behaviour and factors that could facilitate predator searching to be exploited in pest management strategies. For both aspects, it is important that researchers appreciate the context-sensitivity of the different behaviours, and work to understand the influencing cues, triggers, and outcomes that are specific to the species and circumstances involved. In doing so, effective and sustainable agricultural practices can be developed and optimised, particularly for the countries and regions which are under the greatest threat of food security issues. More generally, the use of dropping by the great range of taxa in which the behaviour occurs is underappreciated and has not often been linked to predator search strategies. Future studies addressing these research gaps will build a clearer picture of the behavioural ecology of predator-prey interactions, their consequences for populations and communities, and how these behaviours evolved.

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# Appendix: Detailed biology

## Aphids (Aphidoidea)

Aphids are small (1-10mm long), soft-bodied insects belonging to the superfamily Aphidoidea within the order Homoptera, the plant-sucking bugs (Dixon, 2012). They have a worldwide distribution, though species are more abundant in temperate latitudes compared to the tropics (Sullivan, 2008). Aphids have a head with two compound eyes, a thorax and abdomen, six legs, and are usually plump and ovoid in shape (Sullivan, 2008, see also Figure 2.1). Diagnostic features of their morphology include: a proboscis situated between the forelegs, a pair of siphunculi (also known as cornicles or abdominal tubes) on the dorsum of the fifth abdominal segment that can release a sticky fluid when the aphid is disturbed, a tail-like protrusion at the end of the abdomen called a cauda which can be short and stubby or long and tapering, and antennae extruding from the head, made up of two short and thick basal segments and a thinner flagellum made up of up to four segments (Sullivan, 2008, Dixon, 2012). Some aphids are wingless (or 'apterous') but winged aphids (or 'alates') have membranous wings with the hind wings being much smaller than the fore wings and the fore wings possessing one prominent and one weak longitudinal vein (Sullivan, 2008). Aphids also possess attachment mechanisms associated with their feet that help them to grip various plant surfaces, though these can vary between species in part due to properties of their host plants. Where substrates are rough or coarse-grained tarsal claws can be sufficient, though long bristle-like setae projecting beneath the tarsi may also help (Kennedy, 1986). For other species, usually those whose preferred host surfaces are smooth, adhesive pads or sponge-like pulvilli between the tarsal claws enable attachment (Beutel and Gorb, 2001, Dixon, 2012). Such structures can be very variable in shape (Friedemann et al., 2014), but all serve as reservoirs for adhesive fluid, a secretion necessary to create surface tension at the air-fluid interface and generate adhesive force (Dixon, 2012). Aphid species further vary in size, ranging from less than 0.1mg to 15mg wet mass (Llewellyn, 1984), and can exhibit a wide variety of body colours, spanning from almost colourless to pale yellow, green, brown, black, or even pink (Tsuchida, 2016). Often, aphids are cryptically coloured, as many aphids living on leaves are green (for example, Figure 2.1) while those living on woody structures tend to be more brown in colour (Dixon, 1973, Dixon, 2012); such cryptic colouration may have evolved in part to reduce the likelihood of predation (Tsuchida, 2016).

The life histories of aphids show great diversity, with the complexity and variability of their annual cycle varying both between and within species (Hales et al., 1997). Generally, there will be a single sexual generation in the year where males and females mate, producing fertilised eggs. All of these eggs will hatch into wingless females, known as foundresses, which subsequently birth live young parthenogenetically (that is asexually, without fertilisation). These young are genetically identical parthenogenetic and viviparous females, though some may be winged (alate forms) while others will be wingless (apterous forms) (Hales et al., 1997, Dixon, 2012). Whether wingless or winged forms are produced varies depending on environmental conditions. Wingless morphs allow for effective exploitation of a favourable and plentiful food resource, while crowding or a deteriorating habitat are strong stimuli for the development of winged alates (Llewellyn, 1984, Hales et al., 1997). The number of offspring produced by each female is very varied (Llewellyn, 1984), but either morph of nymph can have embryos already developing within its embryos – a ‘telescoping’ of generations – and this combined with parthenogenesis enables aphids to achieve very high rates of population increase (Dixon, 2012). The number of parthenogenetic generations depends on a range of environmental factors, including temperature. But in temperate regions winged adults generally birth other parthenogenetic aphids in summer and start to produce pre-sexual and sexual morphs of males and females capable of mating and egg-laying in the autumn (Llewellyn, 1984, Hales et al., 1997). Species for which parthenogenetic reproduction is interrupted by annual sexual reproduction are known as holocyclic (Llewellyn, 1984, Sullivan, 2008). Whether aphids are born live from viviparous parthenogenetic females or from an egg following mating they follow the same developmental sequence: usually four instars or stages of being a nymph, then adult (Sullivan, 2008). For some aphid taxa, an alternation of host plants is an additional, obligate part of their annual cycle. For these aphids, known as heteroecious species, ‘primary hosts’ (often a woody plant) are where eggs are laid, then the parthenogenetic generations migrate to ‘secondary hosts’ (usually herbaceous plants), and finally the sexual generations return to the primary host (Hales et al., 1997). Monecious species, by contrast, remain on one host species throughout the year (Sullivan, 2008).

When it comes to colonising a host plant aphids are either generalist, in that they can feed on a large number of host plant species or even families, or more host-specific, requiring certain species to feed successfully – host-specificity can be particularly important with regards to the primary host when aphids are host-alternating (Hales et al., 1997). Whether generalist or host-specific, most – if not all – species of aphids feed on the phloem sap of plants, which is rich in sugars but relatively poor in amino acids (Dixon, 2012). Once a plant is accepted as a potential host, aphids scan its surface with the tip of their proboscis and probe into the plant's internal structure with their mandibular and maxillary stylets – together these form a hollow needle-like structure for the uptake of plant sap (Sullivan, 2008, Dixon, 2012). A continuous salivary sheath is then secreted that gives rigidity to the stylets as they penetrate the plant tissue and take an intercellular route to the phloem (Llewellyn, 1984, Dixon, 2012). Usually, there is more active translocation of amino-nitrogen-rich phloem to parts of plants which are actively growing, to meet the plant's requirements for growth, and this is often where aphids tend to colonise and aggregate (Llewellyn, 1984). Interestingly, the gregarious nature of some aphids means that their feeding can sometimes itself form an 'artificial sink' in plants, wherein there is an increase in phloem sap translocation to the feeding area, similar to that seen in growing regions. Greater translocation translates to better nutrition for aphids and subsequent increases in their growth and reproduction (Llewellyn, 1984). Aphids are entirely dependent on their diet to acquire nitrogen, an element necessary for growth, and so they need to feed continuously, process large quantities of food, and use the nitrogen it contains effectively in order to maintain their very high rates of growth (Dixon, 2012). However, as their diet is sugar-rich but poor in amino-nitrogen, excess sugar is ingested to obtain scarce nitrogen. This results in the production of honeydew, a sugary mixture of nitrogenous and faecal excretory material that is secreted from the anus and often sticks to aphids' feeding surfaces (Llewellyn, 1984). Aphids also harbour the obligate (also known as 'primary') bacterial endosymbiont *Buchnera aphidicola*, which – in return for a stable niche and supply of nutrients – supplements aphids' unbalanced diets by synthesising essential amino acids, vitamins, and sterols (Clark et al., 2010, Skaljac, 2016). Aphids can also host a range of facultative 'secondary' symbionts, which are not essential for survival but can influence fitness in a number of different ways (Clark et al., 2010) – these are discussed further in Chapter Four.

Under suitable environmental conditions aphids can complete their life cycle in a very short time, primarily due to their rapid larval growth, small adult size, and parthenogenetic, viviparous reproduction (Llewellyn, 1984). These qualities enabling short generation times also mean that population sizes can increase rapidly, which can be catastrophic for the yields of agricultural crops that serve as host plants for aphid species (Hales et al., 1997). Aphids can damage plants directly, by nutrient drain when feeding, or indirectly by a number of means including transmission of viruses and coating with honeydew, which can act as a medium for moulds or fungi or attract other pest species (Hales et al., 1997, Sullivan, 2008, AHDB, 2015). However, the abundance and gregariousness of aphids – as well as the relative immobility of their larval stages – also mean they play another important ecological role; a valuable food source for other animals (Llewellyn, 1984). Many predators are associated with aphids, including syrphid larvae (hoverflies), plant bugs, lacewing larvae and adults, and coccinellid larvae and adults. They are also important hosts for parasitoid wasps (Llewellyn, 1984, Sullivan, 2008). The potential of such natural enemies to reduce aphid numbers can be an important aspect of pest regulation strategies, but aphids have evolved a range of antipredator mechanisms that can mitigate such suppression; aphid antipredator behaviours are discussed in greater detail throughout this thesis, particularly in Chapters Four and Five.

There are over 4000 species of aphid (Sullivan, 2008, Dixon, 2012), but the species primarily used in this thesis – pea aphid, *Acyrthosiphon pisum* (Harris) and potato aphid, *Macrosiphum euphorbiae* (Thomas), both in the tribe Macrosiphini (Stekolshchikov and Buga, 2020) – are two economically important pests of agricultural crops (Sullivan, 2008). The pea aphid is a large, usually green aphid (though there are red-pink morphs) with a pear-shaped body (see Figure 2.1), and long antennae, legs, and cornicles (Sullivan, 2008, AHDB, 2015). Alate females grow to 2.5-3mm long and have deep red or black eyes and yellowish-green antennae, while apterous females have similar features but with a smooth and shiny skin (AHDB, 2015). Pea aphids do not host alternate (monecious) and, instead, live on herbaceous plants (including legumes such as alfalfa, clover, peas, and broad beans) throughout the year. In temperate regions they are holocyclic, undergoing several generations of parthenogenetic and viviparous reproduction before reproducing sexually in autumn prior to egg-laying (Llewellyn, 1984, Sullivan, 2008). The eggs overwinter on forage crops, where they remain until hatching in the spring (AHDB, 2015). The primary predators of pea aphids are ladybirds and syrphid larvae (Ximenez-Embun et al., 2014), but other natural enemies include spiders, parasitoid wasps, and fungal pathogens (AHDB, 2015).

Similar to pea aphids, potato aphids are a relatively large, pear-shaped aphid species, with wingless adults reaching 2.5-4mm long (AHDB, 2015). They have long legs, antennae, cornicles, and cauda and, like pea aphids, can also be found as both green and red-pink morphs (Sullivan, 2008). But potato aphids have a characteristic dark longitudinal stripe running down their dorsal surface and winged adults usually have a yellowish-brown head and green thorax (AHDB, 2015). In temperate regions potato aphids are a host-alternating species (heteroecious), producing eggs to overwinter on primary host plants including rose, weeds, and potato sprouts (AHDB, 2015, Xu and Gray, 2020). In spring, the eggs hatch and winged forms migrate to secondary host plants including a wide range of field crops and vegetable crops (AHDB, 2015). Under summer conditions with high temperatures, one female adult potato aphid can produce 20-50 nymphs that mature in 14-21 days; nymphs periodically develop into alate adults and migrate to other plants (Xu and Gray, 2020). A migration back to the primary host takes place for sexual reproduction and egg-laying (AHDB, 2015, Xu and Gray, 2020). Natural enemies of potato aphids include: ladybirds, lacewings, parasitoid wasps, predatory flies, spiders, ground beetles, rove beetles, and insect-pathogenic fungi (AHDB, 2015).

## Ladybirds (Coccinellidae)

Ladybirds, also known as Coccinellidae, are an ecologically and morphologically diverse family of flight-capable small beetles from the order Coleoptera (Seago et al., 2011), that are common wherever aphids are found in the world (Sullivan, 2008). Adults range in length from 1mm to over 10mm, have domed bodies, short, club-shaped antennae, and usually distinctive colourful markings (Sullivan [2008], AHDB [2015], see also Figure 2.2). Even their eggs are easily recognisable, usually coloured yellow or orange, elongate, oval-shaped and laid in batches (Sullivan, 2008, AHDB, 2015). Ladybirds adhere to surfaces using a range of mechanisms, including tarsal claws, dense setae that form adhesive pads, a glandular swelling at their posterior end (known as the pygopodium), and adhesive fluid (Dixon, 2000, Moon et al., 2012, Peisker et al., 2013, Heepe et al., 2016). Like other Coleopterans, adult ladybirds possess tough forewings (known as elytra) which conceal well-developed hind wings used for flight beneath (Kukalová-Peck and Lawrence, 1993). In light or ‘non-melanic’ morphs, the pattern of the elytra usually consists of dark spots on a light background while in dark or ‘melanic’ morphs, the elytra usually has light spots against a dark background (Sloggett and Honěk, 2012). Lighter colours are typically bright yellows, oranges, or reds (AHDB, 2015). The mainly aphidophagous Coccinellinae subfamily are generally patterned with two strongly contrasting colours, while coccidophagous subfamilies lack bright patterning and are mainly black or brown (Dixon, 2000). For different species, various colour morphs and particular patterns may be adapted for different functions. Bright colours are often thought to function as an aposematic ‘warning’ in poisonous species, which can exude noxious alkaloid-based compounds when disturbed (called ‘reflex bleeding’) and are repellent or toxic to their predators (Dixon, 2000, Sullivan, 2008, Seago et al., 2011, Sloggett and Honěk, 2012). Multiple chemically-defended species are often found with similar bright colouration, presumably mutually benefitting from Müllerian mimicry wherein predators more readily learn to avoid such patterned prey. Batesian mimics that imitate warning colouration but are themselves harmless to those predators may also exist, though apparent cases of this may sometimes in fact be cases of different protective alkaloids being selected for effectiveness against different predators (Dixon, 2000, Sloggett and Honěk, 2012). Depending on species and context, colouration could additionally, or alternatively, serve adaptive functions of: crypsis in particular environments, signalling for mates, or as a means of thermoregulation given that different colours vary in their reflectance and absorption of radiant energy (Sloggett and Honěk, 2012).

Ladybirds exhibit a broad trophic diversity, encompassing herbivory, pollenophagy, fungivory, and highly specialised predation on aphids and other invertebrates (Seago et al., 2011). While predation is the primary means by which ladybirds acquire food, supplementary food such as pollen provided by host plants can be important to coccinellid communities, particularly the larvae of certain species (Honěk, 2012). Most species of ladybird are generalist and polyphagous, feeding on a rather wide range of prey and foods, while relatively few are monophagous, specialising and successfully developing on only a narrow range (Hagen, 1962, Hodek and Evans, 2012). Aphidophagous coccinellids tend to lay eggs nearby aphids and their larvae actively seek out colonies of aphids, especially the very mobile final instar (Ferran and Dixon, 1993). When ladybird larvae first hatch from eggs, they often cannibalise other eggs or smaller sibling larvae due to their need to feed almost immediately in order to survive (Sullivan, 2008). Larvae are voracious, particularly in their fourth instar, and as they moult and increase in size they can sometimes consume more than 100 aphids of various species per day (Sattar et al., 2008, Sullivan, 2008, Hodek and Evans, 2012). Larvae have pointed jaws with which they pierce aphid cuticles and inject saliva; this then digests the body contents into a semi-liquid that the larvae suck up (Hagen, 1962, Sullivan, 2008). Piercing and sucking is often accompanied by periodic regurgitation of the contents back into the prey’s body (Hagen, 1962). Later larval stages which have grown and developed a chewing action may also consume solid remains of prey (Hagen, 1962, Sullivan, 2008). The success of ladybird larvae in capturing prey has been found to depend on a range of abiotic and biotic factors, including larval instar, hunger level and genetic characteristics, plant structure, species of aphid attacked, and intra- and interspecific competition (Ferran and Dixon, 1993). Adult predatory ladybirds have powerfully built mandibles that are used to crush and tear their prey. Coccidophagous ladybirds tend to have a unidentate tooth at the tip of each mandible, which they use to cut open their prey's chitinous coverings or prise their prey off a surface, while aphidophagous usually have a bidentate tooth at the tip of each mandible (Dixon, 2000). The mandibles of some aphidophagous species, though, come to a sharp point with a groove down the inner margin and function like hypodermic needles. These species will feed in a manner similar to larvae in that they grab an appendage of their prey with their mandibles before injecting enzymes and sucking out the body tissues once they have been digested (Dixon, 2000). The consumption rates of adult ladybirds can vary with sex and stage in the reproductive cycle, relative size of both predator and prey, prey density, plant surfaces, temperature and humidity (Hodek and Evans, 2012, Honěk, 2012).

Considering the efficiency of foraging, ladybird searching for prey was once commonly believed to be a random process, with neither larvae nor adults reported as perceiving their prey until making contact with it (Hagen, 1962). A tendency towards negative geotaxis and positive phototaxis in both coccinellids and aphids was thought to enhance the efficiency of such random search (Ferran and Dixon, 1993). However, visual and olfactory cues are now thought to be important for foraging (Nakamuta, 1984, Obata, 1986, Lambin et al., 1996, Harmon et al., 1998, Pettersson et al., 2008, Lim and Ben-Yakir, 2020), and plants may provide additional tactile and chemical stimuli when infested with prey (Ferran and Dixon, 1993, Pervez and Yadav, 2018, Norkute et al., 2020). The sensory abilities of ladybirds are still relatively poorly understood, though (Pervez and Yadav, 2018). As do other insect predators, ladybird larvae and adults forage for aphid prey both extensively and intensively. Extensive search involves relatively fast linear movement, while intensive search – which is switched to following contact with or consumption of prey – involves more frequent turns (Ferran and Dixon, 1993). In terms of where ladybirds search, coccinellids often concentrate foraging along the prominent veins of leaves where aphid density is commonly highest (Banks, 1957, Dixon, 1959, Wratten, 1973); this focus on high prey density areas is not always the case as, where leaves lack such veins, the edges of leaves and the petiole are usually searched most (Carter et al., 1984, Ferran and Dixon, 1993). Ladybird larvae in particular have also been reported to waste time revisiting areas where there are no prey present (Banks, 1957, Wratten, 1973). Ladybird searching behaviour is discussed in more detail in Chapters Seven, Eight and Nine.

The life cycle of ladybirds usually lasts around one year, although occasionally there are two generations in a year (AHDB, 2015). As with other holometabolous insects (those with complete metamorphosis), the developmental stages start with an egg, which hatches into a larva that goes through four instars, before it pupates and metamorphoses into an adult (Dixon, 2000). Aphidophagous ladybirds usually lay their eggs in clusters, while coccidophagous ladybirds lay their eggs singly, but once hatched they go through the same number of larval instars (Dixon, 2000). In temperate climates, eggs of aphidophagous ladybirds are usually laid in spring to early summer, pupation occurs mid-summer, and adults emerge in mid-late summer (AHDB, 2015). Developmental time of immatures is highly dependent on both temperature and food quality (Hagen, 1962, Dixon, 2000) but, with sufficient food and warmth, two generations can occur due to fast larval development (AHDB, 2015). Larval development of aphidophagous ladybirds occurs on aphid-infested stands of field crops, wild herbs, shrubs or trees, and young adults then feed on both insect and vegetable food in temporary habitats (Honěk et al., 2007). Before reproducing themselves, these adults typically overwinter in sheltered places (known as hibernacula) with their own microclimate, like leaf litter, dry vegetation, grass tussocks, bark crevices and buildings (Honěk et al., 2007, AHDB, 2015) – often long-distance flights are involved in reaching these sites (Hagen, 1962). When overwintering, environmental conditions are unfavourable (colder and lacking available prey) and adults largely refrain from feeding and enter a dormant state of variable physiological arrest, which can range from simple quiescence to intense diapause depending on species (Hagen, 1962, Hodek, 2012). The ephemeral nature of ladybirds' food supply likely led to the selection of mechanisms enabling long dormancy periods for survival in the absence of suitable food (Hagen, 1962). Hibernating adults may mass together to form aggregations, ranging in size from a few to tens of thousands of individuals (Honěk et al., 2007). If individuals are attracted to particular microclimatic conditions and/or particular structures then aggregations may arise passively, but it is likely that aggregations often originate from an intrinsic tendency to clump together alongside conspecifics, even when other suitable hibernacula are available (Honěk et al., 2007). An aggregation differs from a cluster of competing individuals if individuals are in close contact with each other (Hagen, 1962). There are several potential means by which adults could actively form aggregations, including pheromones and visual cues, but the precise mechanism remains unknown at present (Honěk et al., 2007, Hodek, 2012). As to why aggregation occurs, clustering could decrease individual risk from predation, but high population density also facilitates the spread of disease (Honěk et al., 2007). The principal basis for aggregating seems to be the bringing together of the sexes (Hagen, 1962). Aggregations disperse in response to increasing day length in the spring (Honěk et al., 2007), when adults seek out mates (AHDB, 2015). Ovaries mature once the adults have fed on aphids after the hibernation, but the disbanding of aggregations appears to involve simple dispersals rather than long migrational flights. Mating can occur at aggregational sites or at the feeding grounds after dispersal (Hagen, 1962).

Considering the ecological role aphidophagous ladybirds perform, their suppressing effect on prey populations has been of great interest to scientists and agriculturalists alike. The higher the density of aphids available, the quicker the development and greater the reproduction of ladybirds (Hagen, 1962). Their relationship with, and ability to reduce, aphid populations has led to ladybirds playing an important role in the development of biological control (Hagen, 1962). Biological control involves the use of natural enemy populations to target pest populations and can involve: introduction of enemies, short-term mass releases of enemies, and/or modification of an environment to protect or enhance existing enemies (Orr and Lahiri, 2014). Due to their relatively fast development and high consumption rates, ladybirds are often more effective predators than other generalist predators such as lacewings (Hindayana et al., 2001, Alhadidi et al., 2018). Ladybirds are the taxa behind the most famous case of biological control (Dixon, 2000), in which *Novius cardinalis* (formerly known as *Rodolia cardinalis*) native to Australia was introduced to California in order to control the cottony cushion scale *Icerya purchasi* in 1888-9. The scale threatened the Californian citrus industry, but the ladybird quickly became established and caused a spectacular decline in the pest population (Coulson et al., 2000). However, there are also many cases where they have not proved effective, and often aphidophagous coccinellids are less successful control agents than coccidophagous coccinellids (Dixon, 2000). Such pest suppression failures can result from the partial synchrony of aphidophagous ladybirds and aphids’ life cycles stopping ladybirds from preventing pest numbers rising and/or their tendency to disperse from areas to which they are introduced (Dixon, 2000). Besides their interactions with prey, ladybirds are also ecologically important as a food resource for their natural enemies – these include insects in the orders Diptera and Hymenoptera, as well as birds (Sullivan, 2008), and parasitoid wasps (Ceryngier et al., 2012).

The family Coccinellidae comprises roughly 6000 species (Seago et al., 2011), but the species primarily used in this thesis – seven-spot ladybird, *Coccinella septempunctata* and two-spot ladybird, *Adalia bipunctata* – are two of the most common found in Britain (Roy et al., 2012). As its name suggests, the seven-spot ladybird is typically characterised by a red elytra with seven black spots (see Figure 2.2). *C. septempunctata* is thermophilic, found mainly on herbaceous plants, and is the most common ladybird in Europe (Ferran and Dixon, 1993, Dixon, 2000, Hodek and Michaud, 2008). Its large size and sun-loving lifestyle might put it at greater risk from birds compared to insect predators (Dixon, 2000), but the alkaloid coccinelline it possesses is very effective against some bird predators (Marples et al., 1989). *C. septempunctata* is an important biological control agent, feeding on many different species of aphid (Rana and Kakker, 2000, Sattar et al., 2008), but also other insects, fungal spores, and pollen (Triltsch, 1997). Unfortunately, there are cases of attempted biological control where it has gone on to decimate native species of coccinellid after introduction (Elliott et al., 1996).

Like *C. septempunctata*, the two-spot ladybird also lives up to its name sometimes, but it is a highly polymorphic species occurring in many forms ranging in colour from red to black (Omkar, 2005). The most common melanic forms are black with either four or six red spots, and the most common non-melanic forms are red with two black spots or red with either two irregular black patches or with two large black spots, with one or more small satellite spots each (Omkar, 2005). *A. bipunctata* is typically a tree-dwelling species (Omkar, 2005), but can be found foraging on weed and vegetable plants (Sengonca et al., 2002) or other shrub and herbage habitats (de Jong et al., 1991, Ferrer et al., 2016). Interestingly, while most ladybirds are usually protandrous (where the first mating of males occurs at an earlier age) (Hodek and Honek, 1996), there is a tendency towards protogyny (where the first mating of females occurs at an earlier age) in *A. bipunctata* (Hemptinne et al., 2001). This might allow females to store sperm ahead of sexual maturation, which would be advantageous given that *A. bipunctata* has overlapping generations and the sexes would benefit from emerging at the same time (Hemptinne et al., 2001). *A. bipunctata* are regarded as Batesian mimics of *C. septempunctata*, but they themselves produce large quantities of reflex fluid rich in the alkaloid adaline (de Jong et al., 1991) and so may not entirely rely on mimetic deception for protection (Dixon, 2000, Omkar, 2005). Two-spot ladybirds are smaller and more secretive in lifestyle than seven-spot ladybirds and, therefore, may experience relatively less predation from birds (Dixon, 2000). Instead, their adaline could be more effective against certain insect predators (Agarwala and Dixon, 1992), or help to reduce hymenopteran parasite loads (de Jong et al., 1991). When acting as a predator itself, *A. bipunctata* feeds on many aphid species as well as coccids, diapsids, and pollen (Omkar, 2005), but has been found to be a relatively poor biological control agent. Despite some limited successes when utilised as part of combined releases with other natural enemies, efforts have often failed due to unfavourable abiotic and biotic conditions such as poor weather, male-killing symbionts, and victimisation by avian and coleopteran predators (Omkar, 2005).

Both *C. septempunctata* and *A. bipunctata* are polyphagous and generalist, eating a wide range of aphid species encountered in their habitats: *C. septempunctata* can feed on at least 24 aphid species (Hodek and Michaud, 2008), while *A. bipunctata* can feed on at least 50 aphid species (Ferrer et al., 2016). Prey suitability for a given predator is thought to vary depending on such factors as: generational exposure to prey, efficiency of prey capture, palatability and ease of ingestion, the presence of allelochemicals (secondary plant metabolites), and nutritive value (Omkar, 2005). Different aphid foods can have species-specific impacts on ladybird larval development speed, prey consumption rates, and adult fecundity, with sub-optimal prey causing reductions in some or all of these areas (Blackman, 1967). For different aphid species, the relative predatory efficiency of seven-spot and two-spot ladybirds can also differ (Deligeorgidis et al., 2011). Nonetheless, *C. septempunctata* and *A. bipunctata* have both been reported to predate pea aphids (Alhadidi et al., 2018, Alhadidi et al., 2019) and potato aphids (Walker et al., 1984, Sengonca et al., 2002).

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