Parasite-induced warning colouration

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Rebecca Susan Jones

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Preface

I hereby declare that this thesis consists of my own work, unless otherwise stated, and has not previously been submitted to the University of Liverpool or any other university in application of a higher degree. The text does not exceed 100,000 words and meets the formatting requirements of the University of Liverpool.

Contents

Abstract

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Parasites are ubiquitous in nature and are capable of exerting strong selection pressures on their hosts to enhance (or potentially reduce) transmission. Parasite manipulation of hosts can therefore drive evolution of various traits and phenotypes in the host to the benefit of the parasite. These adaptations can serve a number of purposes, working to enhance survival and reproduction of the parasite within its host. This thesis aims to elucidate the roles of various defences induced by the nematode *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens* in its obligate insect host, in which predation of the host is fatal for the parasitic colony. To do this I utilised both laboratory and field experiments to test a number of the defences with a variety of predators. To begin with I extended a previous study examining predation rates on uninfected and infected individuals by examining the effect of background on predation rates in the field. I found that prey that were conspicuous against their background received fewer attacks and were consumed less than those that were cryptic with respect to their background, enhancing survival for the parasitic colony within infected hosts. Following this I was then able to test a number of the defences utilising ground beetles, birds and mice as predators. In a laboratory setting I tested whether beetles could use any of the parasite-induced cues to avoid predation of infected waxworm hosts. I found infections were vulnerable early on (day 3 post-infection) in terms of chemical defence as beetles would consume this infection stage to a greater extent than either day 5 or 7 post-infection waxworms. However, beetles utilised the olfactory cue to avoid predation of infected hosts across all infection stages, protecting the parasite colony. Having seen an effect of the visual cue, and perhaps olfactory cue in the initial field experiment, I decided to test both these components in concert and singly in a laboratory environment with wild-caught great tits in Finland. There was not a clear benefit to multimodality in terms of attacks but there was in terms of consumption of infected waxworms at various stages of infection. Additionally, there was evidence that the olfactory cue overshadowed the visual cue in terms of attack at various stages of infection. Having examined the visual, chemical and olfactory cues, I then tested the role of bioluminescence in this nematode-bacterium system. Utilising house mice as predators I tested both the olfactory cue and bioluminescence cue with the same experimental design under differing light conditions, where the bioluminescence was and was not visible. Unlike in other predators tested, the olfactory cue did not elicit a strong avoidance response, resulting in only discriminatory behaviour towards later stage infections (day 7 post-infection). However, I found that bioluminescence was an effective cue at causing deterrence in house mice as mice spent less time near glowing than non-glowing prey. Overall, this thesis provides novel insights into the role of defences induced by a nematode-bacterium complex in protecting the infected host carcass against predation, which is fatal for the parasitic colony. Furthermore, the thesis provides ideas for future research to develop these findings further.

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Chapter 1. General Introduction

1.1 Introduction

In order to fully appreciate the questions posed in this thesis, it is important to understand two main fundamental concepts, in seemingly disparate areas, underlying this work, namely the concepts of aposematism and host manipulation. By understanding and combining ideas from both of these areas, it is possible to understand the experiments carried out and their rationale. In this introduction I therefore discuss both aposematism (1.2) and host manipulation (1.3) and how these ideas fit together (1.4). Additionally I also introduce the study system in depth (1.5) and the aims of the thesis (1.6).

1.2 Aposematism: An introduction

Almost all animals are targeted by predators during their lifetime so it is not surprising that there is a wide range of anti-predator strategies to reduce the likelihood of being eaten. The range of these defences is diverse and operate at various stages of predator-prey interactions (Stevens, 2013). One anti-predator defence, protective colouration, can be divided into primary defences, acting before a predator attacks, and secondary defences, operating during or after an attack (Edmunds, 1974). Primary defences can include camouflage, warning signals and mimicry (Stevens, 2013).

Alfred Wallace, in correspondence with Charles Darwin, originally suggested that conspicuous colouration of Lepidopteran larvae could have been utilised to alert predators to the presence of toxins (Wallace, 1867). This relationship between

warning colouration and toxicity then became known as aposematism (Poulton, 1890). Aposematic prey therefore have defensive properties, making them unprofitable to prey, which they advertise with warning colouration (Cott, 1940; Edmunds, 1974; Poulton, 1898). Aposematism is now the term utilised to describe the anti-predator strategy whereby conspicuous or distinctive signals across sensory modalities are utilised to warn predators of a chemical defence across a wide range of taxa (Mappes, Marples, & Endler, 2005). Aposematism, most prolifically found in insects, has also been demonstrated in molluscs (e.g. nudibranchs), reptiles (e.g. coral snakes), amphibians (e.g. dendrobatid frogs), fish (e.g. puffer fish) and mammals (e.g. skunks) (Blount, Speed, Ruxton, & Stephens, 2009).

Evolution of aposematism

The initial evolution of aposematism is a conundrum and there are three suggested routes of evolution, starting from a cryptic population of profitable prey (Guilford, 1988):

1) Prey become unprofitable and then evolve a conspicuous signal to advertise this

This evolutionary route is considered the most likely (Alatalo & Mappes, 1996; Guilford, 1988; Härlin & Härlin, 2003; Harvey & Paxton, 1981; Tullberg, Leimar, & Stille, 2000). This is hypothesised to occur when a small mutation leads to the production of a defence, such as consumption of a new toxic food plant or the ability to store toxins. Once this defence has arisen, and if it confers a fitness benefit to the bearer, then it is expected that it will spread through the population. If this new defence is effective then it would be expected that a further signal,

potentially bright colouration, that increases detection rate, memorability or easy recognition will increase survival as predators attack fewer conspicuous prey.

2) The conspicuous signal appears first and then prey become unpalatable

This evolutionary route is considered improbable by many (Guilford, 1988; Harvey & Paxton, 1981; Riipi, Alatalo, Lindström, & Mappes, 2001; Stuart-Fox, Marples, Kelly, & Thomas, 2005; Moussalli, Marshall, & Owens, 2003) as conspicuous advertisement of palatable prey seems unlikely to be beneficial. This suggests that there will be a cost to being conspicuous whilst the meaning of the signal is being established, perhaps leading to extinction of the early individuals with this colour mutation.

3) Unprofitability and conspicuous colouration arise simultaneously

This idea was initially discounted as the likelihood of the two mutations required for unprofitability and conspicuous colouration arising at the same time are extremely small (Guilford, 1988). However, it has been theorised that if a cryptic individual were to move to a new food plant, they may appear more conspicuous on this food plant. Additionally, if this food plant then had a toxin which the individual was able to sequester and store, then the increased conspicuousness would be able to signal this to predators (Lindström, Alatalo, Mappes, Riipi, & Vertainen, 1999; Ruxton, Sherratt, & Speed, 2004).

All of these routes require a novel colour morph to persist long enough to spread through the population (either defended in route 1 and 3, or not in route 2). The survival and spread of novel conspicuous morphs is considered an obstacle as it is assumed that the more conspicuous prey will be consumed first (Alatalo &

Mappes, 1996; Gittleman, Harvey, & Greenwood, 1980; Harvey & Paxton, 1981; Riipi et al., 2001; Stuart-Fox et al., 2003). Although there is some evidence for selection against novel colour forms (Gittleman & Harvey, 1980; Gittleman et al., 1980), there is increasing evidence for selection favouring novel colour forms.

A number of studies have found that avian predators often avoid novel insect prey (Coppinger, 1969, 1970) and even that conspicuous plumage in songbirds reduces the risk of attack by birds of prey (Gotmark, 1992, 1993, 1994, 1996). Additional evidence demonstrates that when a bird encounters a novel form of a prey type in a population with familiar prey types, it will avoid the novel ones (Marples & Kelly, 1999; Marples, Roper, & Harper, 1998; Thomas, Bartlett, Marples, Kelly, & Cuthill, 2004; Thomas, Marples, Cuthill, Takahashi, & Gibson, 2003). This long-term avoidance of novel prey has been termed dietary conservatism (Marples et al., 1998) in distinction from neophobia (Barnett, 1958) which is a short-lived phenomenon (lasting a few minutes) to approaching anything new. Dietary conservatism could therefore facilitate the evolution of warning colouration as if predators avoid novel prey for long enough then a novel colour morph could potentially spread through a population and persist, rather than swiftly going extinct (Coppinger, 1969, 1970; Gotmark, 1994, 1996; Marples et al., 2005; Marples et al., 1998).

There is still therefore debate over which evolutionary route led to the evolution of aposematism, although dietary conservatism may enable novel colour morphs to persist long enough for route 2 to be plausible. It is still possible for the evolution of aposematism to occur through three routes: 1) signal then unprofitability, 2) unprofitability and then signal and 3) unprofitability and signal simultaneously.

Effects of aposematism on predator cognition and behaviour

Warning signals are considered to have 'special effects' on predator cognition and behaviour. One of these effects includes how predators initially respond to novel aposematically coloured prey (Gamberale & Tullberg, 1998; Roper & Cook, 1989; Rowe & Guilford, 1996; Schuler & Roper, 1992). There is evidence that birds, including domestic chickens, *Gallus gallus domesticus*, zebra finches, *Taeniopygia guttata*, pheasants, *Phasianus colchicus*, and starlings, *Sturnus vulgaris* have unlearnt aversions to specific colours and colour patterns of artificially modified prey (see review in Schuler & Roper, 1992). These studies, alongside others (Roper, 1990; Schuler & Hesse, 1985; Wiklund & Jarvi, 1982), demonstrate that naïve, as well as experienced predators, learn to avoid novel conspicuously coloured prey. Furthermore, avoidance is normally seen towards classic aposematic colours such as plain black, plain red and black-and-yellow stripes (Roper & Cook, 1989; Roper, 1990; Schuler & Hesse, 1985; Schuler & Roper, 1992) but not plain yellow, olive green or half-black/half-yellow (Roper & Cook, 1989).

Another special effect of aposematism is how predators learn to associate warning signals with toxicity (Gittleman & Harvey, 1980; Ham, Ihalainen, Lindstrom, & Mappes, 2006). There is evidence that predators learn to avoid unpalatable prey more quickly with a warning signal present (Alatalo & Mappes, 1996; Gittleman et al., 1980; Lindström et al., 1999; Riipi et al., 2001; Roper & Wistow, 1986). Additionally, it appears that predators are able to recognise unpalatable prey more quickly (Guilford, 1986) if they are conspicuous (as in the case of warningly coloured individuals), rather than cryptic (Speed, 2000).

Predators also seem to remember the association between warning signals and unpalatability for longer if prey is more conspicuous (Ham et al., 2006; Roper & Redston, 1987; Roper, 1994). Roper & Redston, (1987) found that chicks learnt to avoid conspicuous beads faster than cryptic beads and that avoidance of the conspicuous beads lasted longer. Also, experiences with unpleasant unpalatable prey tends to lower future attack probabilities (see Speed, 1993) and generally increases attack probabilities on palatable prey (Speed, 2000).

Aposematic individuals also benefit from the predators' ability to generalise learned avoidance of a signal to another similar signal (Alatalo & Mappes, 1996; Darst & Cummings, 2006; Duncan & Sheppard, 1965; Gamberale-Stille & Tullberg, 1999) and even avoid new signals, though lacking experience. This is an important mechanism in Batesian mimicry, whereby predators generalise avoidance of warningly coloured prey, i.e. the 'model species', to the similar but perfectly palatable 'mimic species' (Bates, 1862).

Furthermore, in populations where automimicry occurs, the presence of indistinguishable palatable individuals in an unpalatable, aposematic prey species, automimicry renders aposematism unstable (Guilford, 1994). When automimics are rare they benefit from predator avoidance of the warning signal, without bearing the associated cost of unpalatability, and subsequently increase in the population (Gamberale-Stille & Guilford, 2004). However, as mimic numbers increase, predator attacks increase due to lower efficacy of avoidance learning, resulting in attacks on unpalatable, as well as palatable prey, according to their frequencies (Gamberale-Stille & Guilford, 2004; Jones, Davis, & Speed, 2013; Skelhorn & Rowe, 2006). Guilford, (1994) however proposed the idea that aposematic signals may function as 'go-slow' signals, rather than 'stay away'. Therefore, if prey is handled with care and

the predator is able to determine the palatability of a prey item at relatively little cost, then automimics and automodels will be distinguishable. It would still benefit the automodel to invest in toxins and signal this conspicuously if predators do little damage to unpalatable prey, but eat palatable prey (Guilford, 1994).

Predators however can sometimes benefit from consuming aposematic prey when the cost of ingesting toxins is outweighed by the benefit of obtaining nutrients (Rowland, Mappes, Ruxton, & Speed, 2010; Sherratt, 2003; Sherratt, Speed, & Ruxton, 2004; Speed, 1993). This idea is supported by laboratory experiments whereby chicks selectively ingest unpalatable individuals based on their own toxin burden (Skelhorn & Rowe, 2007) which suggests that warning signals may function as honest signals of toxicity (Blount et al., 2009). Predator cognition and behaviour therefore have profound effects on the evolution and maintenance of aposematism in prey populations.

Multimodal signalling

Many animals produce and react to displays made up of multimodal signals. A multimodal signal is one where components of the signal occur in more than one sensory modality (Rowe, 1999; Scheffer, Uetz, & Stratton, 1996). Although much of the focus has been concerned with multimodal signalling in a sexual signalling context (Hebets & Uetz, 1999; Scheffer et al., 1996), there has been an increase in studies on this complex signal design in other fields. Examples include aggressive displays (Anderson, DuBois, Piech, Searcy, & Nowicki, 2013) and warning signals (Marples, van Veelen, & Brakefield, 1994; Siddall & Marples, 2008). Stemming from this, there is now a plethora of hypotheses surrounding the evolution and function of

multiple signals within different contexts (Candolin, 2003; Hebets & Papaj, 2005; Partan & Marler, 1999).

These hypotheses (see review in Rowe & Halpin, 2013) cover both content and efficacy based hypotheses. Some relate to how multiple signals can increase information value of a signal, the 'multiple messages' or 'back-up' signal hypotheses (Moller & Pomiankowski, 1993). Others relate to how signal components evolve in response to variability within the environment (Candolin, 2003; Hebets & Papaj, 2005) or the perceptual variability in predators relying on signal components in different sensory modalities (Rowe & Halpin, 2013). Multicomponent signalling can also lead to increased detection (Rowe, 1999), improved discrimination (Hebets & Papaj, 2005) and increased learning and memory (Siddall & Marples, 2008). Multimodal signals have also been suggested to act in a sequential manner due to the unique properties of different sensory modalities that make signals more detectable at different distances or environmental conditions (Candolin, 2003; Hebets & Papaj, 2005).

Toxicity is one of the most studied and common forms of defence utilised by aposematic prey but there is evidence that warning signals can also combine multicomponent features, such as taste and smell, and these seem to accelerate learning if colours are novel (Marples, van Veelen, & Brakefield, 1994; Marples & Roper, 1996). Aposematic, or warning signals, therefore provide a wide range of examples of multimodal signalling (Rowe & Guilford, 1999). Many studies have examined how odour and/or sound interact with warning colouration (Eisner & Grant, 1981) to deter predation in domestic chicks (*Gallus gallus domesticus*) utilising artificial combinations of various cues (Marples & Roper, 1996; Rowe & Guilford, 1996, 1999; Siddall & Marples, 2008). The combination of multiple cues often results

in an increased latency to consume novel prey or faster avoidance learning compared to either cue alone (Marples & Roper, 1996; Siddall & Marples, 2008). For example, Siddall & Marples (2011) found that wild robins (*Erithacus rubecula*) learnt to avoid artificial pyrazine (a common insect warning odour) treated yellow baits faster compared to those with no odour. However, it is vitally important to understand how these results translate into the natural environment using wild predators (Siddall & Marples, 2011) and natural aposematic prey (Marples et al., 1994). To our knowledge the only study examining multimodal signalling effects of a naturally occurring aposematic insect is that by Marples et al., (1994) whereby the authors tested various combinations of the multimodal signal of the seven-spot ladybird (*Coccinella septempunctata*). Ladybirds were presented to captive Japanese quail (*Coturnix coturnix japonicas*) in treatment combinations with colour pattern, scent and taste singly, in a two-way combination or the whole insect. Avoidance was maximised when the whole insect was presented, although colour was the most effective single deterrent (Marples et al., 1994).

Conclusions

Throughout this thesis I then refer to aposematism as the combination of a warning colour, odour or sound with a chemical defence, usually a toxin. It is a widespread anti-predator defence strategy in a range of taxa, though most commonly found in insects. Although the evolutionary route to aposematism is still debated it is thought to have evolved in one of three ways: unprofitability then signal, signal then unprofitability or signal and unprofitability simultaneously. Aposematism however has profound effects on predator cognition and behaviour as predators

respond to novel aposematically coloured prey, learn to associate warning signals with toxicity, remember these signals and then generalise them across other similar signals, as in the case of Batesian mimicry. Warning signals however do not only combine a warning colour and a toxin, but are often multicomponent. Various studies have examined the effects of sound and odour on avoidance and learning of aposematic cues, suggesting that when combined, a multimodal signal may provide the greatest avoidance and latency to attack.

1.3 Parasite manipulation

Parasites often have complex life cycles with only a small probability of surviving and reaching maturity and so have developed several characteristics which appear to increase the probability of completing their life cycle (Poulin, 1994). This is usually achieved through either high fecundity, host location mechanisms by infective stages or asexual multiplication at one stage of the life cycle (Poulin, 1994). Furthermore, many parasites are capable of manipulating their host's behaviour which will aid them in completing their life cycle (Dobson, 1988). From the first empirical demonstration of larval ancanthocephalan parasites infecting amphipods, causing aberrant behaviour and abnormal colouration making them more susceptible to predation by the parasite's next hosts (Hindsbo, 1972; Holmes & Bethel, 1972), there has been sustained interest in parasite manipulation. As a result, parasite manipulation has been well documented in a couple of hundred host-parasite interactions spanning all major phyla (see review in Moore, 2002).

Most of the known cases of parasite manipulation involve subtle changes in one aspect of host behaviour or appearance, but some are truly remarkable

manipulations. Two trematode species have become textbook examples as both require an intermediate host, where parasites develop as larvae, to be ingested by a definitive host, normally a predator of the intermediate host (Poulin, 2010). One species, *Dicrocoelium dendriticum*, is transmitted through accidental ingestion of ants by sheep. It causes infected ants to climb to the tips of grass, attach themselves and wait for grazing sheep (Carney, 1969; Moore, 2002). Another example, *Leucochloridium* spp., causes the antenna of its snail intermediate host to change shape, size and colour, as well as pulsate violently in response to light. This gives the appearance of potential caterpillar prey which attracts birds, the next host for the parasite (see Moore, 2002). Other examples include a nematode parasite which turns the abdomen of its ant intermediate host a bright red in colour and drives the ant to perch in patches of red berries with its abdomen raised. Here, they are predated by frugivorous birds which act as definitive hosts for these nematodes (Yanoviak, Kaspari, Dudley, & Poinar, 2008).

Parasite manipulation therefore is usually defined as a parasite-induced alteration in the host's phenotype resulting in fitness benefits for the parasite (Poulin, 2010). This generally means that infected hosts behave in a manner that facilitates transmission of the parasite to complete its life cycle. Therefore, the phenotypic traits induced by the parasite in the host are either directly or indirectly modulated by genes in the parasite genome, one of the main concrete examples of the extended phenotype proposed by Dawkins (1982).

What is adaptive manipulation?

Three alternative explanations have been proposed for the resultant change in an organism following infection by a parasite (Poulin, 2010). Firstly, the change may arise due to specific actions of the parasite on the host, altering its behaviour to benefit the parasite. For example, trematodes causing ants to climb to the tips of grass, where they are consumed by sheep (Carney, 1969). Secondly, the change may result from an adaptive response of the host to parasite infection, either trying to eliminate or negate the effects of infection. For example, the cytoxic defence system of coral polyps turns polyps pink in reaction to invading trematodes, which ultimately benefits parasite transmission into the definitive host (Aeby, 2002). Thirdly, the change in host behaviour may be a by-product of pathology that by chance may benefit parasite transmission. It is only scenario 1 that can truly be called "adaptive manipulation". In particular, Poulin, (1995) suggested four basic criteria that had to be met for apparent manipulation to be considered adaptive:

1) Complexity

Simple changes may have arisen by chance or the by-product of other selective changes. However, complex traits are unlikely to be due to chance, and so require an organising principle such as natural selection (Poulin, 1995). For example, for ants whose abdomen turns bright red and perch among red berries following nematode infection, this change seems too complex and too well fitted to parasite transmission to have arisen by chance (Yanoviak et al., 2008).

2) Purposiveness of design

Changes in host behaviour must show some conformity to *a priori* expectations based on their predicted function. For example, the onset of behavioural changes would be expected to coincide with the developmental stage which would benefit the

parasite the greatest in terms of transmission (Poulin, 1995). Larval stages of trophically transmitted parasitic worms often only induce behavioural changes in their intermediate host once they are developmentally ready to be transmitted into their next host (Bethel & Holmes, 1974).

3) Convergence

Convergence on the same behavioural manipulation between unrelated parasite lineages is suggestive of parasite manipulation, rather than chance (Poulin, 1995). Two different and unrelated phyla, mermtihid nematodes (Nematoda) and hairworms (Nematomorpha), require a terrestrial arthropod host to enter water, where the parasite can emerge (Poinar, 1991). For this to have evolved independently twice in distinct lineages suggests some sort of adaptive function.

4) Fitness benefit

Ultimately the most important criterion to meet is that the adaptive trait must confer a fitness benefit to its bearer; therefore parasites altering their host's behaviour must achieve greater transmission than those who can't (Poulin, 1995). Although this has only been confirmed in a small number of documented cases, it provides the best evidence of adaptive manipulation. Mouritsen & Poulin (2003) found that manipulation of the trematode *Curtuteria australis* in the New Zealand cockle (*Austrovenus stutchburyi*) led to a greater number of parasites being transmitted to the target host, compared to those that weren't manipulated.

In my opinion, and others (Poulin, 2010), these criteria now seem too strict to apply to cases of adaptive manipulation as it is really only the last criterion, conferring a fitness benefit to the bearer, which is important. Furthermore, it is often

difficult to distinguish between an advantageous by-product and an advantageous direct product of selection (Poulin, 2010). For example, coral polyps infected with the trematode *Podocotyloides stenometra* turn bright pink following infection and this increased visibility causes them to be eaten preferentially by their definitive host, butterfly fish (Aeby, 2002). This pigment is produced by a protein involved in the host's cytotoxic defence system (Palmer, Roth, & Gates, 2009) and although harmless to the trematode, is actually beneficial for its transmission. You would therefore expect selection to favour parasites that would induce more pronounced colour changes in their host. Therefore, for parasite manipulation to be considered 'adaptive', there must be a genetic basis to the change in host behaviour which ultimately leads to enhanced transmission (Poulin, 2010).

How do parasites manipulate their host?

At a taxonomic level, parasite manipulation has been documented in most of the major lineages of parasitic organisms and is thought to have evolved at least 20 separate times among parasite lineages (Poulin, 2010). At an ecological level, the vast majority of parasites utilise one of four general transmission routes:

1) Trophic transmission

In trophic transmission, the larval or juvenile stage of a parasite is transmitted from its intermediate host to its definitive host by predation. This usually consists of altering the appearance or behaviour of the intermediate host to increase its susceptibility to predation by a suitable definitive host (Lafferty, 1999). Many parasitic

worms with complex life cycles, such as trematodes, cestodes, acanthocephalans and nematodes utilise this type of manipulation.

2) Host movement

The second type of transmission is normally observed where parasites must either exit their 'current' host, or release their propagules, in a habitat other than the one where that host lives in order to facilitate transmission to an alternative host (Poulin, 2010). In this case, the parasite normally alters the behaviour of the host so that it moves to a different habitat, sometimes one that is completely unsuitable for the host. For example, the nematomorphs and mermethid nematodes, discussed earlier, both cause their terrestrial arthropod host to seek out and jump into water so the parasite can complete its life cycle as eggs and larva of each develop in aquatic environments (Poinar, 1991).

3) Vector-borne transmission

Vector-borne transmission involves pathogens transmitted between vertebrate hosts by blood-sucking insects, such as mosquitoes. The parasites are picked up by one vector through the blood meal and then transmitted to another in a subsequent blood meal. Parasite transmission depends on the number of hosts visited by a vector and so parasite manipulation is utilised to shorten the duration of individual blood meals and increase the number of hosts visited (Moore, 1993). Parasites utilising this strategy include viruses, protozoans such as trypanosomes and *Plasmodium spp*. and filarial nematodes (Moore, 1993)

4) Parasitoid transmission

The fourth type of transmission is most commonly used by insect parasitoids, Hymenoptera and Diptera, which must exit the host following growth inside and pupate on external substrates. Manipulation in this context involves altering the behaviour of the host to protect parasite-infected pupating hosts from predation. This can occur by the host moving to a specific microhabitat prior to emergence (Brodeur & McNeil, 1989), the host being induced by the parasitoid to produce physical structures to protect the emerging parasitoid, for example Ladybirds and *Perilitus spp.*, (Eberhard, 2000) or the host defending the pupating parasitoid against predators (Brodeur & Vet, 1994).

There is also some evidence of manipulation for contact-transmitted parasites, although evidence is either lacking or not that convincing. It has been reported that some sexually transmitted parasites can alter the sexual behaviour of their hosts, leading to increased contacts with mating partners (Abbot & Dill, 2001). Rabies has also been suggested as a form of parasite manipulation as the rabies virus is transmitted by an infected host biting a susceptible host whereby rabid animals often exhibit increased aggression. However, increased aggression is only one of the possible manifestations of rabies (Hemachudha, Laothamatas, & Rupprecht, 2002; Rupprecht, Hanlon, & Hemachudha, 2002). Contact-transmitted parasites might not have been studied explicitly in the context of parasite manipulation and so as this field grows, this may shed new light on this potential route of parasite transmission and manipulation.

Manipulation of host traits

When discussing parasite manipulation, most studies examine the visual changes in colouration, morphology or behaviour but there must be a biochemical or physiological pathway underlying these changes. In some bizarre cases, parasite manipulation results in a completely new behaviour, such as crickets jumping into water. However, most often they target existing behaviours which are manifested by small changes in expression. For example, parasite manipulation may result in a slight shift in the proportion of time an individual carries out a particular behaviour or spends in a certain microhabitat. Some trophically transmitted parasites are also able to modify the behaviour of their hosts to avoid predation when the parasite larva has not yet reached the developmental conditions allowing it to successfully establish in the next host; this is termed predation suppression (Dianne et al., 2011; Médoc & Beisel, 2011; Parker et al., 2014). Predation suppression then becomes predation enhancement as parasites manipulate their hosts to become more susceptible to predation when developmentally ready to be transmitted to the next host.

Parasites are also found to modify basic host tropisms (e.g. responses to light, humidity), reactions to threat stimuli (disturbances or cues associated with predators) or activity levels (see Moore, 2002). Parasites also alter host behaviour through direct or indirect mechanisms, such as interfering with the host's nervous system or muscle (Thomas, Adamo, & Moore, 2005). For example, a parasite may secrete or excrete a neuroactive substance, resulting in changes in host behaviour. There is good evidence that parasites secrete hormones and venoms into their host and that they alter both host development and behaviour (Adams, Alewood, Craik, Roger, & Lewis, 1999; Beckage & Gelman, 2001; Gelman, Kelly, Reed, & Beckage,

1999). For example, the avian schistosome, *Trichobilharzia ocellata*, secretes a substance that induces its snail host, *Lymnaea stagnalis*, to release a neuromodulator to inhibit host egg laying (De Jong-Brink, Reid, Tensen, & Ter Maat, 1999; Hordijk et al., 1992). Therefore resources that would have been allocated to host reproduction are now allocated to parasite reproduction.

Parasite manipulations are frequently known for one particular phenotypic change (e.g. ants infected with the trematode *Dicrocoelium dendriticum* climb to the top of grass to be consumed by sheep, crickets parasitised by hairworms leaping into water for the parasites to complete their life cycle) but it is increasingly recognised that infected hosts are deeply modified organisms with a range of modifications occurring simultaneously and/or successively (Brodeur & Boivin, 2004; Cezilly & Perrot-Minnot, 2005; Poulin & Thomas, 1999; Thomas et al., 2005). One reason for this is that the parasites don't just alter one trait, but several traits of their hosts (Hughes, Brodeur, & Thomas, 2012; Poulin, 2010; Thomas, Poulin, & Brodeur, 2010). Parasite manipulation is considered multidimensional if there are at least two changes in different phenotypic traits, or in the same phenotypic trait (e.g. behaviour, morphology and/or physiology) (Hughes, Brodeur, & Thomas, 2012; Poulin, 2010; Thomas, Poulin, & Brodeur, 2010). However, these must not correspond to measurements of the same alteration. For example, for a behavioural change associated with neurological disorders induced by the parasite, the atypical behaviour displayed and associated neurological basis cannot also be considered.

From a phylogenetic perspective, manipulative parasites are most likely to derive from non-manipulative ones, as it is most parsimonious to assume that the original manipulation involved alteration to a single host phenotype (Poulin, 2010). Therefore, any parasite capable of altering one aspect of its host phenotype resulting

in enhanced transmission would be favoured over conspecifics by natural selection. Furthermore, addition of a novel alteration to a single manipulation is likely to be favoured if the transmission benefits outweigh the extra costs of this additional alteration (Thomas et al., 2010). Therefore, multidimensional manipulations are likely to arise when the interaction between the host alterations boosts the transmission in a synergistic fashion. For example, trophically transmitted parasites can increase susceptibility of its intermediate host to its definitive host by simultaneously altering the behaviour and the colour of its host (Bakker, Mazzi, & Zala, 1997; Sánchez et al., 2009).

Furthermore, multidimensional manipulations can occur simultaneously or sequentially. Whilst the phenotypic changes induced in *Gammarus insensibilis* by the trematode *Microphallus papillorobutus* (positive phototactism, aberrant evasive behaviour (Helluy, 1984)) occur simultaneously, in some parasites the changes occur sequentially. In crickets infected with hairworms, the first behavioural change, erratic behaviour, occurs before the worm is fully mature, increasing the probability of encountering a suitable body of water for worm emergence (Thomas, Poulin, & Brodeur, 2010b). The second behavioural change, suicidal behaviour, then enables the parasite to physically enter the water (Sanchez et al., 2008).

A lot of effort has gone into the study of parasite manipulation, but relatively few studies have considered the multidimensional nature of parasite manipulation. Furthermore, there is encouragement to consider the ecological context whereby multidimensional manipulations might occur (Thomas et al., 2005). Parasite manipulation can be considered in the context of behavioural ecology where complex signal function has well documented the use of more than one display to advertise the qualities of certain individuals in a population (Moller & Pomiankowski,

1993). A number of hypotheses have been suggested to explain why multiple signals have evolved (Candolin, 2003; Rowe, 1999; Rowe & Halpin, 2013) including: the use of redundant signals to ensure information will be transmitted, 'multiple messages' which provide information about different qualities and different signals for different receivers (see 'Multimodal signalling' section earlier). Parasites utilising a multidimensional manipulation could also be considered in this context. Through altering a number of phenotypic traits in their hosts (through the extended phenotype) to aid transmission, these parasites could be viewed as signallers sending multiple signals to other individuals or species, for example, as seen in the effects trophically-transmitted parasites have on their predatory definitive hosts. Therefore, the conceptual framework of multiple complex signalling could be applicable in the context of multidimensional parasite manipulation by parasites.

Endosymbionts as adaptive manipulators

Parasite manipulation is generally considered between parasites, such as trematodes, cestodes, etc. and their hosts but there is also evidence of symbiont-induced manipulation altering morphological, physiological and behavioural aspects of their host. These manipulations can result in increased transmission benefits for the symbiont through their host populations (Hughes et al., 2012). One endosymbiont of particular interest is *Wolbachia*, which is found in a wide range of host species, mostly belonging to the phylum arthropoda. *Wolbachia* infect up to two thirds of all insect species (Hilgenboecker, Hammerstein, Schlattmann, Telschow, & Werren, 2008), as well as mites, spiders, scorpions and terrestrial crustaceans (Baldo, Prendini, Corthals, & Werren, 2007; Bordenstein & Rosengaus, 2005;

Rowley, Raven, & McGraw, 2004; Wiwatanaratanabutr & Kittayapong, 2009). Furthermore, *Wolbachia* are also present in filarial nematodes (see Taylor, Hoerauf, & Bockarie, 2010) and have also been detected in a plant-associated nematode (Haegeman et al., 2009). *Wolbachia* are obligate endosymbionts which are normally transmitted transovarilly with the cytoplasm from infected females to their offspring, although there can sometimes be horizontal transfer through vectors, such as parasitoid wasps (Werren, Baldo, & Clark, 2008).

Wolbachia can be both parasitic and mutualistic and numerous studies have demonstrated that *Wolbachia*-based arthropod manipulation is multidimensional, causing alternative reproductive phenotypes (Saridaki & Bourtzis, 2010 and references therein) and affecting host physiology (Brownlie et al., 2009; Kremer et al., 2009), immunity (Kambris, Cook, Phuc, & Sinkins, 2009) and behaviour (Miller, Ehrman, & Schneider, 2010; Vala, Egas, Breeuwer, & Sabelis, 2004). One of the most commonly described *Wolbachia*-induced manipulations is reproductive parasitism through cytoplasmic incompatibility (CI) which results in embryonic lethality among offspring (Yen & Barr, 1971). CI is an extremely efficient tool for the endosymbiont to promote and secure its own transmission, thus promoting rapid spread and persistence in a population (Hughes et al., 2012). Endosymbionts are therefore capable of altering host phenotype to enhance transmission and reproduction.

Conclusions

Parasite manipulations serve to increase the chance of a parasite completing its life cycle through a number of different routes (Poulin, 1994). There are a number

of transmission routes parasites utilise, with the main focus on altering some aspect of the host's behaviour in order to be predated by a definitive host (Lafferty, 1999). Traditional views of parasite manipulation thought parasites invoked a change in one dimension of the host's phenotype, but it is now accepted that parasites are likely to alter more than one dimension, known as multidimensional manipulation (Brodeur & Boivin, 2004; Cezilly & Perrot-Minnot, 2005; Poulin & Thomas, 1999; Thomas et al., 2005). This therefore has implications in complex signalling as parasites alter a range of phenotypes in their infected hosts to signal to predators, and so should be viewed in the context of multisignal-receiver theory. As well as parasites, there is growing literature surrounding the role of endosymbionts in altering their host's phenotype to increase their chance of transmission. Parasite manipulation of host biology is therefore a widespread and complicated phenomenon observed in many phyla, aimed at increasing the transmission of the parasite into the host where it is capable of reproducing.

1.4 Parasite manipulation through aposematism

Parasite manipulation therefore helps to increase the chance of parasites completing their life cycle through altering some aspects of the host's behaviour in order to be predated by the definitive host (Lafferty, 1999; Poulin, 1994). Of the various transmission routes possible, a large number of parasites are trophically transmitted, meaning that the parasite passes through a number of hosts in order to reproduce. Textbook examples of this include the trematode parasite *Dicrocoelium dendriticum*, which is transmitted to sheep by ingestion of infected ants (Carney, 1969; Moore, 2002) and *Leucochloridum* spp. which alter the shape, size and colour

of snail antenna, giving them the appearance of caterpillar prey to their definitive host, birds, (see Moore, 2002).

A number of parasites however alter the colour of their host in order to be predated by their definitive host (Aeby, 2002; Palmer et al., 2009; Yanoviak et al., 2008), reflecting the use of aposematism as an anti-predator defence although aposematism is utilised to deter predation. Coral polyps infected with the trematode *Podocotyloides stenometra* turn bright pink following infection which is produced by a protein involved in the host's cytotoxic defence system (Palmer et al., 2009). This bright pink colour however increases the visibility of infected polyps, causing them to be preferentially consumed by their definitive host, butterfly fish (Aeby, 2002). Therefore, selection should favour parasites promoting a more pronounced colour change in their host. Another example of host manipulation through an induced colour change is that of the nematode infection of ants, *Cephalotes atratus*, which causes their abdomens to turn bright red (Yanoviak et al., 2008). Infected ants also perch in red berries with their abdomens raised to increase transmission into birds, their definitive host.

Although these examples are not strictly aposematism as they have not been shown to have a chemical defence which backs up the aposematic colouration, there is clearly evidence that parasites can utilise induced colour changes in their host to enhance transmission. It is therefore not a large leap to suggest that in certain parasite species induced colour change might be utilised to avoid host predation, especially in those parasites that are not trophically transmitted and where consumption of infected hosts is therefore detrimental to the survival of the parasite colony. Furthermore, if, as in aposematism, this colour change was then backed up by a chemical defence, either induced by the parasite or as a host response to

negate the effects of infection, then aposematism might be a viable strategy for predator deterrence of infected hosts in which consumption would be fatal for the parasites.

Parasites that may employ this tactic are entomopathogenic nematodes belonging to the family *Heterorhabditidae*. As non-trophically transmitted parasites, infected hosts act as breeding grounds for the nematodes and their symbiotic bacteria, meaning predation of the host is fatal for the parasite colony. In the case of *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens,* infected hosts however undergo a number of host changes: their integument turns red, they bioluminesce, produce a chemical defence and have a foul-smelling odour (discussed in greater detail in sections below) (Daborn, Waterfield, Blight, & Ffrench-Constant, 2001; Ffrench-Constant & Bowen, 2000; Ffrench-Constant et al., 2003). This nematode system therefore has the capability to signal aposematically through the use of the chemical defence, paired with other potential cues, and it is these cues that have been the focus of this thesis.

1.5 Heterorhabditis bacteriophora and its symbiotic bacterium Photorhabdus luminescens

Introduction

The nematode *Heterorhabditis bacteriophora* is an insect parasitic (entomopathogenic) rhabditid nematode which is found in the eurhabditid clade with *Caernorhabditis elegans* (Ciche, 2007). The nematode was first described in 1976 as a new genus and provides a good model for parasitism, symbiosis and vector-borne disease, as well as its use as a biological insect control (Ciche, 2007). This is largely

due to the symbiosis it shares with the bacterium *Photorhabdus luminescens*. This association between *H. bacteriophora* and *P. luminescens* is very similar to that occurring between *Steinenerma carpocapse* and *Achromobacter nematophilus*, another nematode species (Milstead, 1979). To be able to reproduce and parasitise insects a third stage infective juvenile needs to transmit the symbiotic bacteria (Han & Ehlers, 1998). In this description of the nematodes I will focus on the life cycle of the nematode-bacteria system, the symbiosis, the range of insects that can be parasitized and the method of infection.

Life cycle

H. bacteriophora has a non-feeding, developmentally arrested infective juvenile (IJ) stage which is the only stage found outside the insect host (Poinar, 1975). The IJ stage remains in the soil and seeks an insect host, where it then enters the haemocoel, starts to develop (recover) and releases the symbiotic bacteria (Hosseini & Nealson, 1995). The regurgitated bacterium rapidly kill the insect host, usually within 24 hours and the bacterium has an LD_{50} of <10 cells in the haemocoel (Milstead, 1979). Within the haemocoel, the developmentally arrested dauer juveniles (DJ), which are similar to the dauer stage in *Caenorhabditis elegans*, feed on the bacteria and host tissues, developing into self-fertilising hermaphrodites with a female phenotype (Poinar, 1975). These then produce a second generation of amphimictic (sexually reproducing) males and females, as well as DJs (Strauch, Stoessel, & Ehlers, 1994). The amphimictic adults then mate to produce a third generation and it is these individuals that emerge from the insect host (Johnigk &

Ehlers, 1999). Nematode development of neonate, male, female and hermaphrodite stages is described below.

i) Neonate juvenile stage

After hatching, the first juvenile stage (J1) of *H. bacteriophora* has a length of 180µm and a diameter of 20µm (Fig. 1.1.). The body is transparent and sex determination occurs 8 to 12 hours after hatching from the egg (Johnigk & Ehlers, 1999).

ii) Male Juvenile development

At the second stage (J2), the male is a length of 270-300µm with a diameter of 25µm (Fig. 1.). The male characteristics, such as the asymmetric gonad region and the curved tail are easily recognised at this stage (Johnigk & Ehlers, 1999). The third male juvenile stage (J3) has a length of 370-400µm with a diameter of 28-35µm. The posterior section of the body becomes broader. At the fourth stage (J4) all the sexual organs are developed and sperm is present in the seminal vesicle (Johnigk & Ehlers, 1999). Following the last moult to the adult stage, the body length is 640-700µm with a diameter of 40-45µm.

iii) Female juvenile development

The female and hermaphrodite pregonads always develop symmetrically which makes them easy to distinguish from males (Johnigk & Ehlers, 1999). At the centre of the body, the gonad forces the intestine to the dorsal side, creating a half-moon shaped field when observed under a microscope. The tail is a lot thinner and sharper than the tail of males. Additionally, in comparison to the hermaphroditic juvenile stage (J2D), the female (J2) is shorter and broader (Johnigk & Ehlers, 1999). At the

J2 stage, the length of the nematode is 280-320µm with a diameter of 25-30 µm. This then increases to 380-420µm at the moult to the third stage (J3) with a diameter of 30 µm. At the fourth moult (J4) the sexual organs are visible and the first egg descends the uterus. Mating is likely to occur before the last moult to adulthood and sperm can be found in the receptaculum seminis as the first egg descends (Johnigk & Ehlers, 1999). At the adult stage, the length of the female is greatly variable as it depends on nutritional conditions, ranging from 700-3000µm with a diameter of 50-200µm.

iv) Hermaphrodite development

The infective juveniles of *Heterorhabditis sp.* are always developmentally arrested hermaphrodites and so dauer formation and recovery are key events during the development to hermaphrodites (Johnigk & Ehlers, 1999). After the moult to the second stage (J2D) the juvenile hermaphrodite is easily recognisable compared to the female as the predauer development is visible. The length of the J2D stage is approximately 440µm and appears spindle-like, with a thin pharynx and sharp tail (Johnigk & Ehlers, 1999). At the early J2D stage the juvenile is still feeding but this ceases when the intestinal lumen collapses and bacteria are found in the anterior part. The J2D then moults into a young DJ which then elongates, depositing storage vesicles around the anus and pharynx. The DJ leaves the J2D cuticle through the mouth opening (Johnigk & Ehlers, 1999). Egg production occurs before the moult to the adult hermaphrodite and during the moult fertilised eggs travel into the uterus to meet sperm which lies in the curve between ovary and uterus.

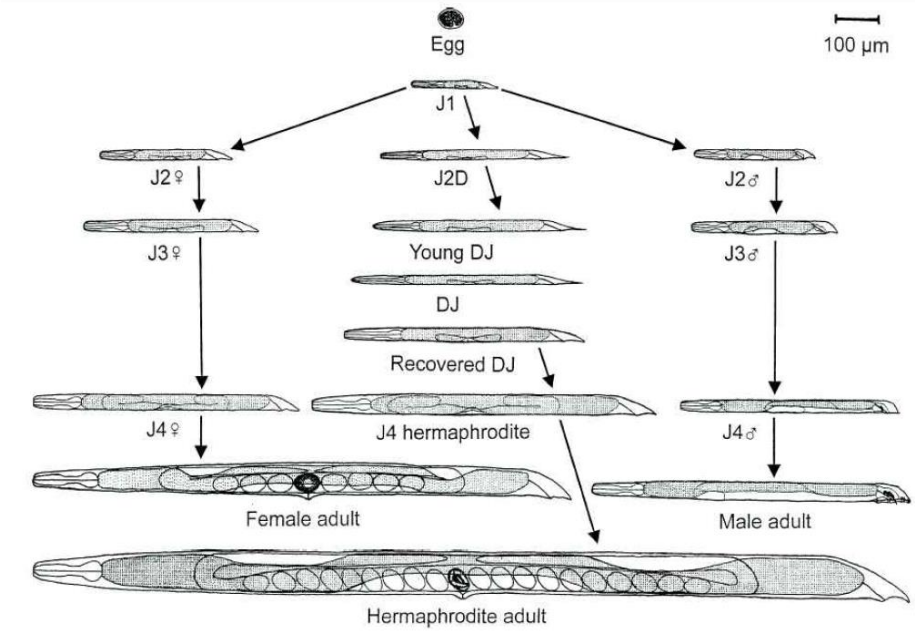

Figure 1.1. Schematic drawing of juvenile (J), dauer juvenile (DJ) and adult development in *Heterorhabditis sp.* with arrows indicating moults between stages. An unfertilised egg can be seen in the female adult, with a juvenile in the hermaphrodite adult. Scale bar is 100µm. Taken from Johnigk & Ehlers, 1999.

Another aspect in the life cycle of the nematode is the process known as *endotokia matricida*. This usually occurs after a number of rounds of egg laying when the adults begin to retain eggs inside their body cavity (Ciche, Kim, Kaufmann-Daszczuk, Nguyen, & Hall, 2008). Once these eggs hatch, the resulting juveniles digest the maternal tissues (matricide) and develop into IJs (Noguez et al., 2012). It is thought that low food availability within the maternal uterus triggers egg retention and IJ formation and may facilitate the transmission of the symbiotic bacteria to the IJs (Ciche et al., 2008). It has recently been discovered however that *H. bacteriophora* secretes a novel ascaroside, asc C11 EA, which prevents this IJ recovery (Noguez et al., 2012). When lower densities of *H. bacteriophora* adults

undertake *endotokia matricida* the IJs that form recover once they emerge from the adult (Ciche et al., 2008). This suggests that asc C11 EA, which most likely increases in concentration at higher nematode densities, may prevent this recovery so that IJs accumulate inside an insect host (Noguez et al., 2012). Furthermore, Ciche et al., (2008), report that the symbiotic bacteria *P. luminescens* are transmitted maternally to IJs via the following sequence of events:

- Adherence to the maternal posterior intestine
- Growth within the intestinal lumen
- Invasion of the rectal gland cells
- Release to the maternal body cavity
- Adherence to the pharyngeal intestinal valve cells
- Invasion of the pharyngeal intestinal valve cells
- Colonisation of IJ intestinal lumen (Ciche et al., 2008).

Recovery

Recovery is the term given to the resumption of development of the dauer juvenile (DJ) into the adult hermaphrodite. Recovery is particularly high in insect hosts with approximately 95% of DJs recovering but in liquid culture recovery varies enormously. This is due to the lower efficacy of the food signal in liquid culture and the lack of host specific cues (Strauch & Ehlers, 1998). DJ recovery in general is therefore induced by either bacterial or insect food signals (Strauch & Ehlers, 1998). However, some members of the population can recover in the absence of a food signal, for example in Ringer's solution (Jessen et al., 2000).

Under *in vivo* conditions the DJ encounter a food signal that immediately induces recovery when they enter an insect host (Strauch & Ehlers, 1998). The response to the food signal from the insect hosts is immediate and complete however the response to the bacterial signal is delayed and highly variable (Ehlers, Lunau, Krasomil-Osterfeld, & Osterfeld, 1998; Jessen et al., 2000). The food signal produced by the symbiotic bacteria is active later during nematode development (Strauch & Ehlers, 1998). In liquid culture, the recovery of the DJ individuals tends to occur only when the media contains the symbiotic bacteria (Strauch & Ehlers, 1998). One of the factors influencing the onset of DJ recovery is the density of the bacterial cells (Strauch & Ehlers, 1998). The signal of the symbiotic bacterium is composed of at least two compounds and a large proportion has a molecular mass of less than 5 kDa (kiloDalton) (Aumann & Ehlers, 2001). It appears that the signal compounds work synergistically rather than in an additive way (Aumann & Ehlers, 2001). Once the nutrients have then been exhausted, a signal inhibits DJ recovery and induces dauer formation (Aumann & Ehlers, 2001).

It is thought that the regulation of recovery in *H. bacteriophora* is similar to that observed in *C. elegans* which utilises converging signalling pathways (Aumann & Ehlers, 2001). The insulin-like dauer recovery pathway is activated by food and temperature and inhibited by the dauer-inducing nematode pheromone (Aumann & Ehlers, 2001). The pathway is activated when host-secreted signals occurring at decreased pheromone levels induce secretion of acetylcholine from an unidentified neuron (Aumann & Ehlers, 2001). This then triggers the secretion of insulin-like signal molecules by binding to the muscarinic acetylcholine receptor of an insulin-secreting cell, triggering the formation of transcriptional outputs (Aumann & Ehlers, 2001). Furthermore, the second pathway (TGF-β-like pathway) is induced by a TGF-

β-like signal and is also inhibited by a pheromone (Aumann & Ehlers, 2001). The transcriptional outputs of both these pathways then activate dauer recovery metabolism (Kimura, Tissenbaum, Liu, & Ruvkun, 1997; Tissenbaum et al., 2000). As a result, atropine, an antagonist of all subtypes of the muscarinic acetylcholine receptor, inhibited recovery in *C. elegans* and *H. bacteriophora* (Aumann & Ehlers, 2001).

There are a number of methods to detect the recovery of DJs in a culture medium which include:

i) Morphological changes – The head region swells, the sheath covering the nematode is lost and the nematodes are slightly enlarged with a more obvious pharynx (Dolan, Jones, & Burnell, 2002).

ii) Microsphere assay – Fluorescent markers ingested into the intestine by recovering DJs provide a marker for the onset of recovery (Dolan et al., 2002).

iii) Analysis of changes in RNA levels using SYTO dyes – The dye SYTO-12 showed specific and reproducible staining in recovering DJs as soon as three hours after the initiation of recovery (Dolan et al., 2002).

Symbiosis

The nematode *H. bacteriophora* is closely associated to *P. luminescens* which is a gram negative, asporous, rod-shaped bacteria (G. O. Poinar, 1975). Bacteria alone however are unable to penetrate the integument or alimentary canal of the insect host and so are dependent upon the nematode, which acts as a vector of the pathogen (Milstead, 1979). Milstead, (1979) showed that exposure to the bacteria

alone had no effect on host mortality and oral doses of bacteria showed only a low mortality (~7%) in a study of 375 larva.

The bacteria are present as a monoculture in the intestine of the DJ stage of the nematode (Endo & Nickle, 1991). The insect mortality observed in *H. bacteriophora* is primarily as a result of the virulence of *P. luminescens* where a lethal dose of 50% can be as low as 30 cells injected into the haemocoel (Poinar, Thomas, & Hess, 1977). Whilst inside the insect cadaver, the *P. luminescens* act as a food source and the nematode offspring are highly specific in obtaining their specific bacterial strain for both growth and reproduction (Akhurst & Boemare, 1990). Little is known however regarding the mechanism for colonisation of the DJ intestine by *P. luminescens* (Ciche & Ensign, 2003). One clue is the presence of three fimbrial homologs located 54bp 5' of *ngrA* which is a gene required by the bacteria to aid the growth and reproduction of the nematodes (Ciche, Bintrim, Horswill, Ensign, & Meg, 2001). Vivas & Goodrich-Blair (2001), reported that a stationary-phase sigma factor homolog, *rpoS*, is required for *Xenorhabdus nematophilus* to colonise the intestine of the nematode *S. carpocapsae*.

Ciche & Ensign (2003) labelled *P. luminescens* by transposing a green fluorescent protein (GFP) gene, within a mini-Tn5 transposon, into the bacterium's DNA to study the transmission of the bacteria. Epifluorescence microscopy shows that the bacteria are located in the anterior region of the lumen, posterior to the basal bulb, and located throughout the intestine (Ciche & Ensign, 2003). It also seems that the bacteria have a limited ability to multiply or spread throughout the intestine during either incubation or ageing of the nematodes. Furthermore, in some 30-day-old or deceased nematodes, swelling of the nematode intestine was observed and the bacteria were located in the entire body cavity (Ciche & Ensign, 2003). This therefore

suggests that the process involved in the localisation of the bacteria in living nematodes is no longer active in deceased nematodes.

Once nematodes are immersed in haemolymph, *P. luminescens* cells begin to migrate towards the mouth of the nematode (Ciche & Ensign, 2003). The bacteria migrate from the intestine, through the pharynx and exit the mouth, suggesting a process of regurgitation in the nematode. There is no movement of the bacteria either towards the anus or posterior region of the intestine (Ciche & Ensign, 2003). In the study by Ciche & Ensign (2003) the DJ nematodes released the bacteria after a 30 minute lag period and continued to release bacteria at a gradual rate for a further 300 minutes. During this period, nematode movement decreased and rapid pumping of the vesicle inside the excretory pore was observed. The average rate of bacterial release was one cell every 2 minutes for 90 minutes, followed by a slower rate of release thereafter.

The bacterial release factor which causes the regurgitation of the bacteria was present in the haemolymph or insect cell culture supernatants which were cultivated from eight or more orders from the phylum Arthropoda (Ciche & Ensign, 2003). The factor was not affected by heat, pronase digestion, Chelex treatment, EDTA addition or melanisation (Ciche & Ensign, 2003). Furthermore, the mechanism of release seems to depend on nematode activity rather than intrinsic to the bacteria.

H. bacteriophora nematodes evade the innate immune system in the larvae of the greater wax moth (*Galleria mellonella*) whilst the *P. luminescens* cells are engulfed by hemocytes and remain in the fat bodies (Dunphy & Webster, 1988). Then, after about 5 hours, the bacteria emerge from the damaged hemocytes and kill the insect quickly (Ciche & Ensign, 2003). By this time, other microorganisms that

may have been carried into the haemocoel might have been destroyed, ensuring the cadaver is mostly devoid of other saprophytic microorganisms. These microorganisms could have a detrimental effect on nematode growth and subsequent colonisation of the intestine by the bacterial symbiont.

Method of infection

In the soil, entomopathogenic nematodes (EPN) such as *H. bacteriophora, H. megidis* and *Steinenerma feltiae* forage for hosts to infect and their response to host cues depends on their foraging behaviour (Grewal, Lewis, Gaugler, & Campbell, 1994). The foraging strategies used depend on models based on the behavioural responses to encountered stimuli that vary in the quality of information they disclose and how the searchers move through their environment (Lewis, Campbell, Griffin, Kaya, & Peters, 2006). Using the second model, foraging strategies fall into two categories; cruise (foraging) and ambush (sit-and-wait) (Eckhardt, 1979; Pianka, 1966). Cruise foragers allocate more time for scanning for resource-associated cues as they move through the environment and actively hunt for prey (Lewis et al., 2006). Ambush foragers on the other hand scan during long periods of stationary activity with short bouts of movement (Lewis et al., 2006). Foraging in general however has a number of constraints, including declining energy reserves and limitations on the life-span of their bacterial symbiont (Akhurst & Boemare, 1990). One nematode similar to *H. bacteriophora* in that it has an association with a symbiotic bacterium is *Steinenerma carpocapsae*. This is an example of an ambush forager as it remains stationary whilst searching and is unresponsive to host cues (Lewis, Gaugler, & Harrison, 1992). *H. bacteriophora* and other *Steinenerma* species, such as *S.*

glaseri, are typical cruise foragers as they move in search for hosts in the soil and are responsive to host cues (Grewal et al., 1994). Cruise foraging, due to active search, is more energetically costly than ambush foraging and so cruise foragers tend to be larger as they store more lipids (Selvan, Gaugler, & Lewis, 1993). Cruise foragers are attracted to cues that indicate the presence of a potential host (Lewis et al., 2006). These cues can vary from volatile cues, cues dissolved in the water film, host cues or cues from the environment (Lewis et al., 2006). Specifically, (E)-beta-caryophyllene from plants damaged by insect feeding have shown increased attraction and infection by *H. megidis* (Rasmann et al., 2005).

Heterorhabditid DJs therefore respond chemotactically to potential insect hosts in the soil (O'Halloran & Burnell, 2003). Although *H. bacteriophora* and *C. elegans* are classed in the same eurhabditid clade, they show different responses to different volatiles (O'Halloran & Burnell, 2003). *Heterorhabditis bacteriophora* responds to a range of alcohols and organic acids but is repelled by other alcohols and pyrazines (Table 1.1). For example, L-lysine and D-biotin which are highly attractive compounds to *C. elegans* were repellent to *H. bacteriophora*. Additionally, the long-chain alcohols which are repellent to *C. elegans*, are attractive to *H. bacteriophora*. Therefore, changes in the length of the carbon chain and the position of the hydroxyl group in the compound can have a great influence upon the chemo-attractiveness of alcohols to *H. bacteriophora* (O'Halloran & Burnell, 2003). Furthermore, carbon dioxide and subliming dry ice also produces a chemotaxic response in the DJs (O'Halloran & Burnell, 2003).

Remote volatile cues are more important for cruise foraging nematodes whereas ambush nematodes respond to cues in a hierarchical order (O'Halloran & Burnell, 2003). The nematode utilises paired amphids on either side of its mouth as

its primary chemosensory and thermosensory organs (O'Halloran & Burnell, 2003). These therefore play a crucial role in search finding in the soil environment. In *H. bacteriophora*, it is the DJs that rely on olfactory cues to find hosts as once inside the host cadaver they inhabit a nutrient-rich broth of bacteria and so do not have to forage for food. Therefore, for parasitic stages inside the host cadaver olfactory cues are not important and they show a weak chemotaxic response to a number of molecules that the DJs find highly attractive (O'Halloran & Burnell, 2003).

Table 1.1 Chemotaxic responses of *H. bacteriophora* DJs to a range of volatile and water-soluble compounds. Taken from O'Halloran & Burnell, 2003.

Attractants
Alcohols
1-pentanol*, 1-hexanol*, 1-heptanol, 2-heptanol, 1-octanol, 2-octanol, 1-nonanol, 2-nonanol, 3-nonanol
Thiazoles/Pyrazines
4,5-dimethylthiazole, 2-isobutylthiazole, 2-methylpyrazine, benzothiazole, 2-acetylthiazole
Organic acids
caproic acid, caprylic acid, methylvaleric acid
Others
carbon dioxide, dry-ice
Weak attractants
Alcohols
2-mercaptoethanol, 1-butanol, 1-propanol, 1-ethanol, 3-heptanol
Others
carbonated water, uric acid†, host assay, hexanal
Neutral compounds
Alcohols
isobutanol, isoamyl alcohol
Ketones
acetone, 2-butanone, 2-pentanone, 2-hexanone, 2-heptanone, diacetyl
Aldehydes
benzaldehyde, valeraldehyde
Pyrazines
acetylpyrazine
Amines
butylamine
Esters
ammonium acetate, isopropyl acetate, isoamyl acetate, ethyl acetate
Others
copper sulphate†, L-cysteine†, dimethyl sulphoxide, paraffin, formamid, zinc sulphate†, diethyl ether
Repellents
Alcohols
methanol, 1-hexanol*, 1-pentanol*
Pyrazines
2,6-dimethylpyrazine, pyrazinamide
Others
L-lysine†, D-biotin†

* Some molecules listed with an asterisk are attractive at high concentrations and repellent at low concentrations.
† These compounds were applied to the agar 120 min before the DJs were added.

Once an insect has been located the nematode then needs to change its behaviour so that it can gain entry into the haemocoel to continue its life cycle. Laboratory studies by Bedding & Molyneux (1982) describe how *H. bacteriophora* individuals penetrate an insect host. The DJs move over the insect cuticle for several minutes to hours before they attempt to penetrate the cuticle (Bedding & Molyneux, 1982). The DJs keep close to the surface and use their head to explore crevices and folds in the insect's cuticle. During this time, approximately one quarter to one third shed their enclosing L2 cuticle (Bedding & Molyneux, 1982). The remaining DJs attempt entry prior to exsheathment and in this case the dorsal tooth supported the rupture of the nematode's sheath. Cuticular penetration was observed in a number of species with the nematode forcing its head into folds, crevices and leg joints of the insect host (Bedding & Molyneux, 1982). Furthermore, no glandular secretions from the nematode were observed during penetration of the insect cuticle. Then, once the cuticle of the insect has been ruptured, penetration by the nematode normally occurs within minutes (Bedding & Molyneux, 1982). The head enters first and there is then a period of exploration inside the host, followed by penetration of 20-100µm deep into the host. Once one DJ had ruptured the cuticle, others would enter through the same wound (Bedding & Molyneux, 1982). However, bacteria can be carried on the outer cuticle of the DJ and this could potentially infect the host (Poinar, 1979). This is normally avoided by the shedding of the outer cuticle prior to exsheathment and so the symbiotic bacteria are released into a virtually aseptic haemocoel which allows it to dominate the bacterial flora after the insect dies (Bedding & Molyneux, 1982).

Other routes of entry include the mouth opening and anus as insects' mandibles may crush nematodes to death (Gaugler & Molloy, 1981). However, frequent defecation by the insect may expel nematodes attempting entry through the anus and so in some insect hosts, such as grubs and sawfly, mouth entry is more successful (Georgis & Hague, 1981). Another route of entry is through the tracheal system via the spiracles although some species exclude invaders through this method by sieve plates (Lewis et al., 2006). Some nematodes also use the gonad openings of adult arthropods as an entry point, for example ticks (Samish & Glazer, 1992).

When entering the haemocoel the DJ come across the non-self response of the immune system of the host insect (Lewis et al., 2006). The host insect uses encapsulation or activation of a phenol oxidase cascade as a defence against the invading nematodes (Gillespie, Kanost, & Trenczek, 1997). Insects also make use of Toll-like receptors that detect PAMPs (Pathogen Associated Molecular Patterns) which activate microbial peptides (Lemaitre, Reichhart, & Hoffmann, 1997). Non-cellular capsules are formed readily and often consist of melanin, however insects infected by *H. bacteriophora* don't turn black and so *H. bacteriophora* suppresses this mechanism and are not encapsulated (Peters & Ehlers, 1997). Encapsulation of the host depends on the nematode-host species combination and nematodes are not normally encapsulated in a host which is similar to those they naturally infect (Lewis et al., 2006).

Once the infected host has been killed they can remain in or near the soil for between 7-20 days before the next generation emerges from the host and so they may be utilised as a food resource (Lewis et al., 2006). However, field studies have shown that nematode-killed insects were only partially consumed or not consumed at

all by workers of the Argentine ant, *Linepithema humile* (Baur, Kaya, & Strong, 1998). The deterrence of the ants is due to a factor produced by the bacterial symbiont called an ant-deterrent factor (ADF) (Zhou, Kaya, & Goodrich-Blair, 2002). In relation to this, larvae infected with *H. bacteriophora* undergo a major colour change as the infected dead insect turns a pink/red colour and becomes bioluminescent (Ffrench-Constant & Bowen, 2000). The bioluminescence only lasts for a short period at the start of infection, but the colour change remains throughout the infection. There are a number of hypotheses for this colour change and one suggests that the colour change acts to reduce predation as the dead infected insects remain turgid and may be utilised as a food resource. Fenton, Magoolagan, Kennedy, & Spencer, (2011) demonstrated that infected larvae were rarely handled by avian predators and were often rejected if handled. It therefore indicates that the colour change observed acts as a visual deterrent to avian predators to reduce predation. Another hypothesis suggested for this colour change is that it is a by-product relating to the elimination of reactive oxygen species that build up in the host (Ffrench-Constant et al., 2003).

Host Range

H. bacteriophora are known to infect a wide range of different host insects both in the soil and in the laboratory (Table 1.2).

Table 1.2. Host range and specificity of *H. bacteriophora*, indicating stage infected, buccal apparatus utilised, trophic category and importance of infection. Taken from De Doucet, Bertolotti, Giayetto, & Miranda (1999).

Order Family Species	Stage	Buccal apparatus[a]	Trophic category	Importance	*H. bacteriophora*
Anoplura					
Pediculidae					
Pediculus humanus capitis L.	A	S	hematophagous	sanitary	+++
	N	S	hematophagous	sanitary	+++
	E	—	no feeding	sanitary	—
Coleoptera					
Chrysomelidae					
Chrysodina sp.	A	CH	phytophagous	harmful	++
Coccinelidae					
Eriopis connexa Ger.	L	CH	predatory	beneficial	+++
	A	CH	predatory	beneficial	+
Hippodamia convergens Guer.	A	CH	predatory	beneficial	+
Curculionidae					
Naupactus cinereidorsum Hust.	A	CH	phytophagous	beneficial	—
Meloidae					
Epicauta adspersa Klug.	A	CH	phytophagous	harmful	+
Melyridae					
Astylus atromaculatus Blanch.	A	CH	phytophagous	harmful	—
Tenebrionidae					
Tenebrio molitor L.	L	CH	phytophagous	harmful	+++
Diptera					
Culicidae					
Culex pipiens S.	L	CH	detritivorous	sanitary	—
	P	—	no feeding	sanitary	+
Muscidae					
Musca domestica L.	A	S	omnivorous	sanitary	+++
Trypetidae					
Ceratitis capitata Wiedemann	P	—	no feeding	harmful	+++
Hemiptera					
Coreidae					
Pachylis argentinus Berg.	N	S	phytophagous	harmful	+++
Nabidae					
Nabis sp.	A	S	predatory	beneficial	+++
Pentatomidae					
Dichelops furcatus F.	A	S	phytophagous	harmful	+++
not determined	A	S	phytophagous	harmful	+++
Piezodorus guildinii Went.	A	S	phytophagous	harmful	+
Reduviidae					
Dipetalogaster maximus Uhler	A	S	hematophagous	sanitary	—
Triatoma infestans Klug.	N	S	hematophagous	sanitary	+++
Homoptera					
Aphidae					
Acyrthosiphon kondoi Shinji	A	S	phytophagous	harmful	+++
Aphis gossypii Glover	A	S	phytophagous	harmful	—
Lecanidae					
Ceroplastes grandis Hemp.	A	S	phytophagous	harmful	+
Hymenoptera					
Formicidae					
Acromyrmex lundi Guerin	A	CH	phytophagous	harmful	+
Apidae					
Apis mellifera L.	A	CH	pollinivorous	beneficial	+++
Vespidae					
not determined	A	CH	pollinivorous	beneficial	+++
	L	CH	unknown	beneficial	+++
Lepidoptera					
Noctuidae					
Anticarsia gemmatalis Hubner	L	CH	phytophagous	harmful	+++
Heliothis sp.	L	CH	phytophagous	harmful	+++
Rachiplusia nu Gn.	L	CH	phytophagous	harmful	+++
Spodoptera frugiperda Smith	L	CH	phytophagous	harmful	+++
Pieridae					
Colias lesbia F.	L	CH	phytophagous	harmful	+++
Pieraustidae					
Laxostege bifidalis F.	L	CH	phytophagous	harmful	+++
Pyralidae					
Diatraea saccharalis Guagl.	L	CH	phytophagous	harmful	+++
Galleria mellonella L.	L	CH	pollinivorous	harmful	+++
Orthoptera					
Acrididae					
not determined	A	CH	phytophagous	harmful	+++

Note. E, egg; N, nymph; L, larva; P, pupa; A, adult.
[a] Only function considered; CH, chewing; S, sucking.
—, No parasitism.
+, Without development.
++, Until fourth stage larvae.
+++, Adults and IJ production.

In the laboratory studies by De Doucet et al. (1999) the Anopluran order was readily parasitized by both *H. bacteriophora* and *Steinenerma rarum*, but not by *S. feltiae*. *H. bacteriophora* and *S. rarum* parasitized in equal amounts through sucking

or chewing on insect hosts, with values higher than 84%. In this study, the most favourable hosts were lepidopterans and hymenopterans, although other studies suggest lepidopterans and coleopterans are best (Klein, 1990).

Infecting hosts however can be problematic as nematodes routinely infect hosts containing either conspecific or heterospecific nematodes (Lewis et al., 2006). One advantage for the presence of conspecifics is that they may allow outcrossing in future generations and a 'mass attack' of nematodes may be required to overcome the host's defence (Peters & Ehlers, 1997). However, above a minimum number required to mate or attack, each additional nematode then becomes a potential competitor (Lewis et al., 2006). In the wild, as crowding increases, the reproductive output of each invading nematode decreases (Boff, Wiegers, Gerritsen, & Smits, 2000) and no IJs are produced from the cadaver at extremely high densities (Koppenhöfer & Kaya, 1995). However, in a laboratory setting, stenenermatid and heterorhabditid nematodes continue to invade crowded hosts, passing the host's carrying capacity (Lewis et al., 2006). A number of studies have been carried out examining the proportion of nematodes invading over a range of exposure concentrations, with some reporting no change (Ryder & Griffin, 2002) and some noting a decline in nematodes invading with increasing concentration (Boff et al., 2000; Koppenhöfer & Kaya, 1995). This therefore indicates that in these studies some nematodes were capable of detecting and avoiding overcrowded hosts. However, these experiments don't simulate conditions in the field where encounters occur over a longer time frame, and so may not detect mechanisms for avoiding/deterring invasion into crowded hosts. Furthermore, the nematodes' natural hosts may emit signals in response to crowding which may not be observed in the

unnatural wax moth host which is frequently used during laboratory studies (Lewis et al., 2006).

With regards to invading hosts containing heterospecifics, lab and field studies suggest nematodes do not avoid insects that contain another species of nematode. Koppenhofer & Kaya, (1995) demonstrated that *S. carpocapsae* and *S. glaseri* can co-invade *G. mellonella* larvae in the lab and found no effect on nematode numbers in either mixed or single infection experiments. Co-occurrence of steinernmatid nematodes has also been observed in the field. Bovien, (1937) observed the co-occurrence of *S. feltiae* and *S. affine* in bibionid larvae in the field. *Heterorhabditis* and *Steinenerma* species are able to co-infect but are not able to co-exist within a host as *S. carpocapsae* was able to outcompete *H. bacteriophora* unless the heteorhabditid was given a 6 hour lead time (Alatorre-Rosas & Kaya, 1991).

1.6 Aims and Structure

This thesis aims to elucidate the roles of the defences employed by the nematode-bacteria system *Heterorhabditis bacteriophora-Photorhabdus luminescens*. It is interesting to consider why this system utilises more than one potential defence when each one is likely to be costly to generate. Furthermore, the benefit of multiple defences in this system, rather than one large generally acting defence, has not yet been examined. Additionally, the changes induced in the host's phenotype are also induced by the bacterial symbiont, rather than the nematode in an unusual form of parasite manipulation. As an introduction to this thesis, in this

section I will give a brief overview of what each chapter aims to test and how they link together.

Chapter 2 is the first data chapter and builds on the original findings by Fenton et al. (2011) who coin the phrase ‘infectious aposematism’ which considers the combination of visual and chemical defence deterring predation by avian predators. Utilising a similar experimental setup I tested whether there was an effect of being conspicuous or cryptic against a background upon predation rates of infected or uninfected waxworms. I also utilised mealworms as hosts to determine whether host colouration affected predation rates also. I found that although conspicuousness against a background is beneficial in terms of reduced attack and consumption rates by wild birds, crypsis may play a role during the early stages of infection when infections are vulnerable. Furthermore, whether hosts had melanised integuments or not had little effect on predation rates with both waxworms and mealworms attacked to similar degrees.

The next three data chapters then test the different defences in three different potential predator groups. In chapter 3 I tested both the chemical and olfactory defence of infected hosts, by utilising ground beetles as nocturnal foragers. In a laboratory setting I was able to determine predation rates, as well as avoidance, of uninfected and infected waxworm hosts at different levels of infection. Similarly to other studies I found evidence of vulnerability of early stage infections to predation but this might be overcome by the presence of the olfactory cue which was able to protect infected hosts at all stages of infection.

Chapter 4 then aims to tease apart the interaction between the visual and olfactory cues of infected waxworms in a laboratory setting utilising wild-caught great

tits. I visited the University of Jyväskyla Konnevesi research station to test the two cues singly and in concert to determine whether there was a benefit to multimodality within this system. We did not however find an overall benefit to multimodality but found that cues singly were sometimes as effective as both cues together in terms of reducing attack rate.

The last data chapter (5) then examines probably the least understood defence in this system, that of bioluminescence. Bioluminescence occurs early during infection and I was able to elucidate its role through laboratory work with house mice at the Mammalian Behaviour and Evolution group at Leahurst. I first tested for the role of an olfactory deterrent in house mice and then was able to examine the role of bioluminescence utilising a behavioural assay. Contrary to the results observed with ground beetles and birds, I found that mice pay little attention to the presence of the olfactory signal in terms of avoiding infected hosts. However, more interestingly, I was able to show that bioluminescence plays a protective role for early infections in deterring mice from spending time near infected hosts.

Finally, the thesis ends with a chapter on conclusions and future work (Chapter 6). Although discussions of the general concepts are explained in each chapter, the final chapter synthesises these ideas and suggests areas for future research.

In keeping with the requirements of the University of Liverpool, I explain here the role played by co-authors although this is given in greater detail at the start of each relevant chapter under ‘author contributions’. In addition to my supervisors, who provided comments and guidance on the work, there are three co-authors listed. Johanna Mappes (chapter 4) was essentially my supervisor in Finland for three

months whilst I carried out the avian trials in the laboratory environment. Johanna aided with discussion about plausible experimental techniques and provided comments on the manuscript. David Clarke and Jane Hurst (both chapter 5) were responsible for different aspects of the work examining bioluminescence. Dave provided me with strains of nematodes allowing me to conduct experiments and also provided helpful comments on the manuscript. Jane helped devise plausible methods to test for the olfactory and bioluminescence cues, assisted with analysis and provided comments on the manuscript.

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Chapter 2. Conspicuousness against a background protects a nematode-infected host

This chapter is in its final preparations for submission.

2.1 Author contributions

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Andy Fenton and Mike Speed provided comments on the manuscript and discussion of ideas. I designed and carried out the study, conducted the analysis and wrote the manuscript.

2.2 Abstract

Prey use a multitude of defences to avoid predation, of which crypsis is a common form, reducing detectability by matching the background. Much of the focus has been on crypsis in predator-prey systems, despite the fact that other groups, such as parasites, may benefit from interfering with crypsis to increase or decrease transmission into another host. The entomopathogenic nematode *Heterorhabditis bacteriophora* infects soil dwelling larva, causing them to turn red, bioluminesce, produce a strong-smelling odour and a chemical defence. Nematodes reproduce inside infected hosts so predation at any stage is fatal for the parasite. Infected hosts signal aposematically but could also be considered cryptic against their soil substrate. We therefore utilised avian vision models to determine conspicuousness of uninfected waxworms (*Galleria mellonella)* and waxworms at days 3, 5 and 7 post-infection against either a bark or white background before presenting prey to wild avian predators. We also tested infections in mealworm larva (*Tenebrio molitor*) to determine whether colour of the host affected predation rates. We found that avian predators could learn about the distastefulness of prey and also attacked and consumed infected prey to a lesser extent when conspicuous rather than cryptic against their background. However, infected prey were consumed and attacked less compared to uninfected prey on both backgrounds suggesting crypsis may be used at a distance and aposematism at close range to minimise attack on infected hosts. Furthermore, with potential predators with differing visual capabilities infected prey may appear aposematic to some but cryptic to others.

2.3 Introduction

Prey species have evolved many defence mechanisms to avoid predation (see reviews by Edmunds, 1974; Endler, 1986). One of the commonest adaptations is crypsis, where prey match their background so detection is difficult. There are many studies where organisms seem well suited to match their environment (Norris & Lowe, 1964; Sweet, 1985), behaviourally select backgrounds that match their appearance (Endler, 1984; Marshall, 2000) or alter their appearance to changes in their current environment (Greene, 1989; Harper & Case, 1999; McFall-Ngai & Morin, 1991; Messenger, 1997; Stevens & Merilaita, 2009). Cryptic colouration and behaviour are now known to reduce the vulnerability of prey to predators (Bond & Kamil, 2002; Endler, 1978; Robinson, 1969). These studies are based on visual matching, as predators normally seek and capture prey through visual information. However, there is some evidence of chemical crypsis or 'phytomimesis' whereby, for example, caterpillars ingest various plant leaves to alter their chemical cuticular hydrocarbons to avoid detection by ants (Akino, Nakamura, & Wakamura, 2004).

However, crypsis, and various other defences, may be interfered with, for example by parasites which have different fitness requirements from their infected hosts. This is most dramatically seen for parasites with complex life cycles, which transmit between hosts through predation (see Moore, 2002; Rothschild, 1962). Many parasite species alter their host's phenotype to impair crypsis (e.g., by altering host colour, morphology or behaviour) in order to increase conspicuousness and therefore susceptibility to predation, thereby enhancing transmission to those hosts (Bethel & Holmes, 1977; LoBue & Bell, 2011; Moore, 1983, 2002; Wesenburg-Lund, 1931). For example, Seppala, Karvonen, & Valtonen, (2005) found that the

trematode parasite *Diplostomum spathaceum* altered cryptic colouration and cryptic behaviour of infected rainbow trout (*Oncorhynus mykiss*) so that they were more conspicuous to avian predators.

Some parasites however are not transmitted through predation, and only require one host to complete their life cycle, and so do not want their host to be more conspicuous to predators. One example of this is the entomopathogenic nematode *Heterorhabditis bacteriophora* and its symbiotic bacteria *Photorhabdus luminescens*, an obligate and lethal parasite of insects (Stock & Burnell, 2000). *H.* bacteriophora infect soil-dwelling larval hosts, killing them through septicaemia following ejection of their symbiotic bacterium (Stock & Burnell, 2000). Reproduction then occurs within the infected host before new infective juveniles emerge 10-14 days later (Stock & Burnell, 2000), meaning that predation during this time is fatal for the parasitic colony within. The infected host however undergoes a number of changes in the host: turning red, bioluminescing, producing a chemical deterrent and a foul-smelling odour (Daborn, Waterfield, Blight, & Ffrench-Constant, 2001; Ffrench-Constant et al., 2003). Various adaptive values of these phenotypic changes have been suggested but, of particular relevance here, the red colouration has been shown to act as an aposematic warning signal (Baur, Kaya, & Strong, 1998; Fenton, Magoolagan, Kennedy, & Spencer, 2011; Gulcu, Hazir, & Kaya, 2012; Jones, Fenton, & Speed, 2016; Zhou, Kaya, & Goodrich-Blair, 2002). Therefore, instead of increasing the conspicuousness of the host to make it more susceptible to predation, conspicuousness of nematode infected hosts is actually used to warn predators of the unpalatability of infected hosts due to the chemical defence, hence aposematism.

Aposematism is a successful strategy to deter predation by advertising the individual's unpalatability through the use of conspicuous means, such as colour, odours or sounds (Cott, 1940; Edmunds, 1974). Typical warning colours include red, yellow and orange, normally associated with some black patterning which gives maximum visibility against brown and grey backgrounds (Cott, 1940). There are a number of hypotheses as to the benefit of unpalatable prey utilising a conspicuous signal over a cryptic signal which include, but are not limited to: predators learn to avoid unpalatable prey more rapidly if they are conspicuous rather than cryptic (Alatalo & Mappes, 1996; Gittleman & Harvey, 1980; Gittleman, Harvey, & Greenwood, 1980; Lindström, Alatalo, Mappes, Riipi, & Vertainen, 1999; Roper & Redston, 1987); predators remember the association between unpalatability and signal for longer (Roper, 1994) and predators make fewer recognition errors with conspicuous patterns (Guilford, 1986). It therefore seems that signalling unpalatability through conspicuous means is more advantageous than cryptic means in terms of influencing predation cognition and behaviour.

Aposematism induced by *H. bacteriophora* to deter predation therefore seems like a viable strategy to protect the developing infective juveniles in the infected host. However, as soil-dwelling nematodes that infect soil-dwelling larval hosts, turning the infected host red means that infected hosts may actually be cryptic against their soil substrate. We therefore aimed to investigate whether crypsis against the bark background or conspicuousness due to aposematism of infected hosts benefitted infected hosts in terms of reduced predator attacks.

We therefore ran two experiments, the first to examine crypsis and aposematism in *H. bacteriophora*-infected waxworms by placing them on bark

backgrounds (where they were cryptic) and white backgrounds (where they were conspicuous) and their associated predation rates by wild foraging birds. We utilised bird vision models to model how differences between prey and their backgrounds affected visual differences in birds. The second experiment utilised mealworm larva (*Tenebrio molitor*) as more ecologically relevant, melanised prey to determine whether colour of the host had any influence on predation rates.

2.4 Methods

We ran two experiments, the first to examine the effect of background on the conspicuousness and predation of *Heterorhabditis bacteriophora*-infected waxworms. The second experiment was to determine whether predators altered predation rates on melanised hosts, such as mealworm larvae. Experimental field trials and statistical analysis was consistent across both experiments.

Nematode Culturing

Wax worms (*Galleria mellonella*) were infected in the laboratory using standard techniques in which 10 waxworms were placed on filter paper with 1000 IJs/mL of nematode culture (*Heterorhabditis bacteriophora*; Nematop) in a 90mm petri dish (Kaya & Stock, 1997). These infected waxworms were then frozen 72 hours (3 days), 120 (5 days) and 168 hours (7 days) later in a -20°C freezer. These different times were utilised as they showed a progressive colour change from the white uninfected prey to a dark red infected prey with stages in between, had dissimilar spectral colour ranges and were also utilised in the study by Fenton *et al.,*

2011. Uninfected wax worms were also frozen at the same time and kept in a -20°C freezer.

Mealworms (*Tenebrio molitor*) were infected in the laboratory with the nematode (*Heterorhabditis bacteriophora*; Nematop) and frozen 72 hours (3 days), 120 (5 days) and 168 hours (7 days) later. Uninfected mealworms were also frozen at the same time and kept in a -20°C freezer. Prey were frozen to reduce the effects that scent may have during this experiment, though we cannot be certain that any behaviours shown are not also due to an olfactory cue.

Field trials

Three field sites were located at Ness Gardens, Wirral and were baited for avian predators (robins, *Erithacus rubecula,* and blackbirds, *Turdus merula*) with a mixture of sunflower oil and porridge oats (Tesco). Baiting occurred for a period of up to a week, until the oil and oats mixture was consumed overnight. Mealworms were then added to the baiting mixture to allow the birds to acclimatise to the presence of larval prey items at each site, except during the mealworm experiment, where they were baited with waxworms. In both experiments, eight prey each, of days 0 (uninfected controls), 3, 5 and 7 post-infection, were randomly positioned on trays in a grid. Across the four trays, there was a total of 32 prey items, randomly allocated and within 5cm of each other, depending on randomisation. All sites were recorded (BirdCam 2.0, Wingscapes) and observed for 2 hours and any prey attacked i.e. pecked, rejected i.e thrown/dropped or consumed i.e. eaten were noted. Each site was repeated.

For experiments with waxworms, at each site 4 white trays (20 x 35cm) were set up in a rectangular fashion and filled to the rim with bark (Verve large chipped bark, B & Q), the substrate located and utilised at the field sites at Ness. One week after experimental trials on the bark background were complete, the white trays were reversed (turned upside down) so the prey was on a white background. The following year, this experiment was repeated but with the first presentation on the white background, second on the bark background. Trials were carried out on a bark background first (11/03-20/03/13) followed by a white background (25/03-19/04/13). This experiment was then repeated a year later with prey presented on white backgrounds first (17/03-19/03/14) followed by bark (01/04-02/04/14).

Experiments with mealworms were only run on a white background to determine the effect of host colour on predation rates of infected mealworms, compared to waxworms. Trials were carried out on a white background across the three sites from 18/03/15-19/03/15.

Spectrophotometry and visual modelling

To determine whether uninfected and infected individuals were cryptic or conspicuous against their background, the spectral reflectance of uninfected, day 3 post-infection, day 5 post-infection and day 7 post-infection waxworms were tested (all N=100, 6 readings per insect). Additionally, the spectral reflectance of the white tray (*N*=20) and bark (*N*=20) were quantified using an Ocean Optics USB2000 spectrometer, DH-2000-BAL (UV-VIS-NIR) light source and an Ocean Optics WS-1 reflectance standard. Analysis was carried out in Pavo (Maia, Eliason, Bitton,

Doucet, & Shawkey, 2013) utilising an average avian UV system, blue tit double cone sensitivity for luminance and standard daylight.

Statistical Analysis

Data were analysed using binomial glmms (Generalised linear mixed models) run in R (R Core Team, 2013) with attacked, rejected or consumed as the response variable. For the first experiment (*H. bacteriophora* in waxworms) the model was run with background, order of presentation and infection stage, and their interactions, as fixed effects, with site as a random effect. For the second experiment, where background and order were not a factor, the fixed effect utilised was infection stage, with site as a random effect. Graphs were drawn using the predict() function in R.

2.5 Results

Experiment 1: Predation of Heterorhabditis bacteriophora-infected waxworms

Visual modelling

We used the avian visual modelling package Pavo to determine the 'distance' between two colours in units of just noticeable differences (JND) in terms of bird vision. In general, JND values between one and three mean that two colours are unlikely to be discriminated, suggesting a JND of three or above as distinguishable colours (McLean, Moussalli, & Stuart-Fox, 2014). Using the model, we found that infected prey were conspicuous against their white background (day 3; JND=3.25,

day 5; JND=3.65, day 7; JND=4.23) but not against their bark background (day 3; JND=0.51, day 5; JND=0.66, day 7; JND=1.47). The white and bark backgrounds were also visually distinct from each other (JND=3.41). Uninfected waxworms were also not distinguishable from the white background (JND=0.63) and almost were from the bark background (JND=2.83). Hypothetically birds were also not able to distinguish between uninfected controls and day 3 (JND=0.73), day 5 (JND=1.14) or day 7 (JND=1.94) post-infection prey. We can therefore conclude that infected prey of all infection stages were conspicuous against the white background but cryptic on the bark background. Additionally, birds were not able to distinguish between uninfected and infected prey, meaning that any effects were as a result of the levels of conspicuousness against the background.

Experiment

There was a significant two-way interaction between the background prey were presented on and the order of presentation of the different backgrounds on the proportion of waxworms attacked (Fig. 2.1a; including uninfecteds; z=3.569, df=735, p<0.001, excluding uninfecteds; z=2.361, df=551, p=0.018), rejected (Fig. 2.1b; no day 0 rejected; z=2.209, df=735, p=0.027) and consumed (Fig. 2.1c; including uninfecteds; z=2.216, df=735, p=0.0267, excluding uninfecteds; z=1.375, df=551, p=0.169). Waxworms that were presented on a white background first were attacked, rejected and consumed less than those presented on a bark background first. However, this levelled out during the second presentation where prey were attacked, rejected and consumed at roughly equal amounts.

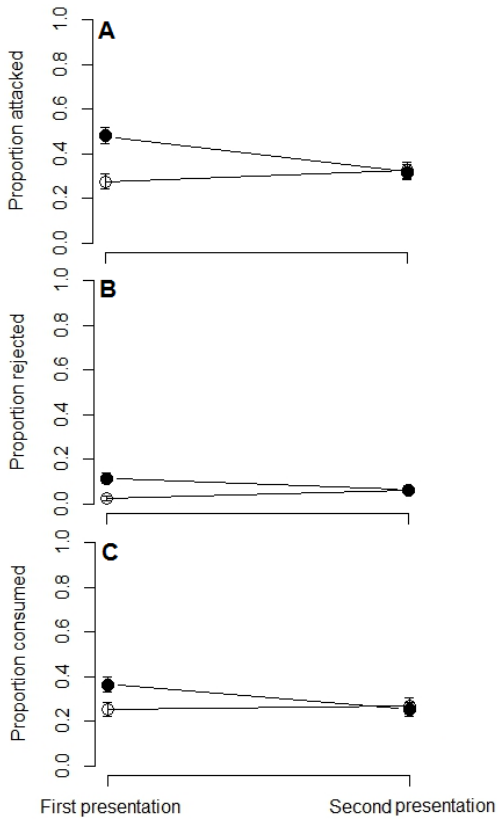

Figure 2.1. Proportion of *Heterorhabditis bacteriophora* infected waxworms a) attacked, b) rejected and c) consumed by avian predators according to background (filled circles= bark background, open circles= white background) and order of presentation (first or second). Error bars represent 95% confidence intervals from the predict() function in R.

There was also a significant two-way interaction between the background prey were presented on and infection stage on the proportion of waxworms attacked (Fig. 2.2a; including uninfecteds; z=-3.571, df=735, p<0.001, excluding uninfecteds; z=-2.511, df=551, p=0.012) and consumed (Fig. 2.2b; including uninfecteds; z=-2.937, df=735, p=0.003, excluding uninfecteds; z=-2.763, df=551, p=0.004). Uninfected prey were attacked and consumed at similar rates irrespective of the background. However, infected waxworms on white backgrounds were attacked and

consumed at lower rates compared on those on bark backgrounds, with a much steeper decline in predation rate as infection stage increased (Fig. 2.2).

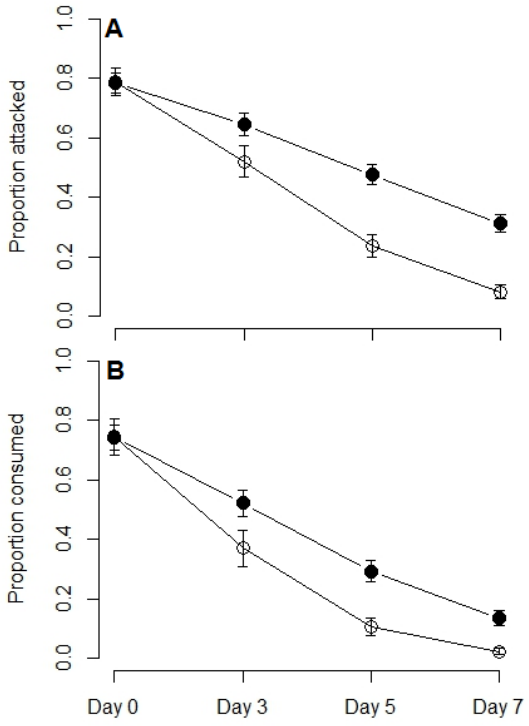

Figure 2.2. Proportion of *Heterorhabditis bacteriophora* infected waxworms a) attacked and b) consumed by avian predators according to background (filled circles= bark background, open circles= white background) and infection stage (Day 0, 3, 5 or 7). Error bars represent 95% confidence intervals from the predict() function in R.

There was a significant two-way interaction between order of presentation and infection stage on the proportion of waxworms consumed (Fig. 2.3; including uninfecteds; z=-2.640, df=735, p=0.0083, excluding uninfecteds; z=-0.876, df=551, p=0.381). Although uninfected waxworms were consumed at similar rates, infected waxworms were consumed less on the second presentation (Fig. 2.3). This suggests

that birds were learning about the infected prey, which is distasteful, and adjusting their responses on the second encounter of infected prey.

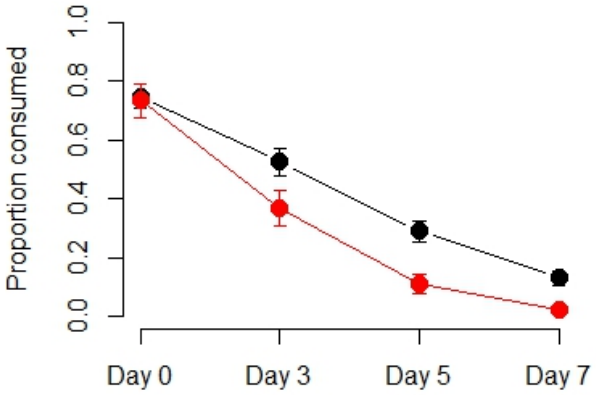

Figure 2.3. Proportion of *Heterorhabditis bacteriophora* infected waxworms consumed by avian predators according to order of presentation (black= first presentation, red= second presentation) and infection stage (Day 0, 3, 5 or 7). Error bars represent 95% confidence intervals from the predict() function in R.

Experiment 2: Predation on Heterorhabditis bacteriophora-infected mealworms

There was a significant effect of infection stage on attack rate including uninfected mealworms (Fig. 2.4a; z=-5.103, df=159, p<0.001) but not excluding them (z=-0.674, df=119, p=0.500), suggesting infected were all attacked at a similar rate. There was also a significant effect of infection stage on consumption rate (Fig. 2.4c; including uninfecteds; z=-5.716, df=159, p<0.001, excluding uninfecteds; z=-4.848, df=119, p<0.001). However, there was no effect of infection stage on rejection rate of the waxworms (Fig. 2.4b; including uninfecteds; z=1.515 df=159, p=0.13, excluding uninfecteds; z=472, df=119, p=0.637). Mealworms were attacked and consumed at relatively equal rates, due to the low level of rejection observed. Uninfected

mealworms were attacked and consumed the most with a decreasing rate as infection stage increased.

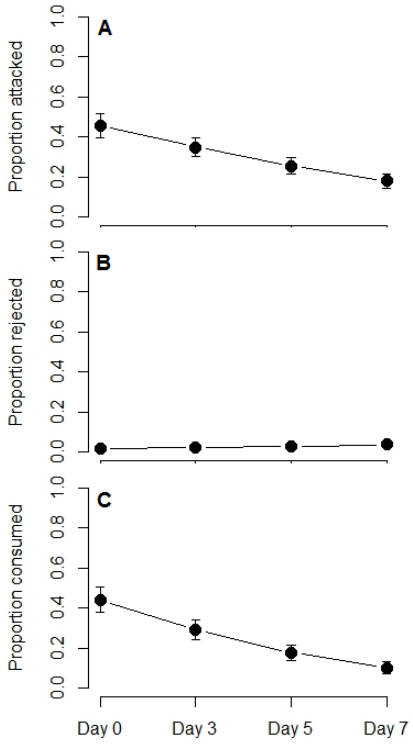

Figure 2.4. Proportion of *Heterorhabditis bacteriophora* infected mealworms a) attacked, b) rejected and c) consumed by avian predators. Birds were presented with 8 mealworms that were either uninfected, or 3, 5 or 7 days post-infection on a white background. Error bars represent 95% confidence intervals from the predict() function in R.

2.6 Discussion

We discuss our results in light of the potential for both aposematism and crypsis to play a role in predator deterrence in this nematode-bacterium system.

Additionally, we examine the effect of host colouration and vulnerability to early stage infections and the roles these play in protecting the parasitic colony within infected hosts.

Benefits to conspicuousness

Our work extends that previously carried out by Fenton et al., (2011), that showed birds would reject infected waxworms over uninfected waxworms on a green background. However, we provided birds with all infection stages simultaneously presented on either a white (contrasting) or bark (approximate colour matching) background and found a number of interactions between background, order of presentation and infection stage.

Waxworms were less likely to be attacked, rejected or consumed if they were on a white background for the first presentation, but this attack, rejection and consumption rates were then about equal on both bark and white backgrounds at the second presentation. This could be due to neophobia towards white trays at the first presentation as birds were trained to feed at sites on a bark background. Furthermore, waxworms were consumed less on the second presentation compared to the first presentation. However, uninfected waxworms (day 0) were consumed at equal rates irrespective of presentation with only infected waxworms (days 3, 5 and 7) consumed less on the second presentation. We know infected individuals contain a chemical defence (Fenton et al., 2011; Gulcu et al., 2012; Jones et al., 2016; Zhou et al., 2002) so these results suggest birds are learning about distasteful infected individuals and lowering their consumption rates on their second presentation of

infected waxworms. Additionally, although we utilised frozen insects to try to minimise the effects of scent, we must consider that some of the effects we see may also be due to the effect of the foul-odour produced by infected insects, which is capable of causing deterrence in its own right (Jones et al., 2016).

What is particularly intriguing is the background and infection stage interaction. Although uninfected waxworms were attacked and consumed at similar rates on the bark and white backgrounds, infected waxworms on white backgrounds were attacked and consumed less, decreasing at a sharper rate as infection stage increased. Infected waxworms were more conspicuous against the white background than uninfecteds (Pavo vision model results) and suffered fewer attacks, suggesting an advantage of a conspicuous rather than cryptic signal. This result reflects a number of experimental studies, mostly conducted in lab settings, highlighting the benefits of a conspicuous signal (Alatalo & Mappes, 1996; Gittleman & Harvey, 1980; Gittleman et al., 1980; Lindström et al., 1999; Roper & Redston, 1987; Sillen-Tullberg, 1985; Tullberg, Leimar, & Gamberale-Stille, 2000). Most of these studies however have utilised artificial prey, whereas here we have utilised live insect prey, and shown that although conspicuousness may be initially costly, it's later beneficial due to avoidance learning. Therefore, it may not be conspicuousness *per se* that is beneficial but the interaction between colour and contrast which enhances the learning process of predators.

Our experiments add to the small number of experiments utilising real (though dead) insect prey to examine crypsis versus aposematism. There are at least two unusual systems whereby prey exists in one of two morphs, a cryptic or conspicuous signal, suggesting either a cost to producing the conspicuous signal or differences in

predation rates (Lindström et al., 1999; Sillen-Tullberg, 1985; Sword, 1999). Sillen-Tullberg, (1985), utilised a red and grey larval form of *Lygaeus equestris* (Heteroptera, Lygaeidae) presented on a grey background whereby the red form was aposematic and the grey form cryptic. Although not a dissimilar setup to our study where the same form was either aposematic or cryptic against its background, aposematic prey had higher survival rates due to greater reluctance to attack, rapid avoidance learning and lower frequency of death given an attack. It would therefore seem to benefit *H. bacteriophora*-infected waxworms to be more conspicuous against their background to reduce attack and consumption rates, to minimise death of reproducing parasites in an infected host. Sword, (1999) also found that grasshoppers (*Schistocerca emarginata*) that lived gregariously in large densities with yellow and black markings had an advantage over the second cryptic, low density morph in terms of predation. These examples, as well as our study, highlight the importance of considering the life-histories of aposematic prey since the animals' colouration represents the end result due to a number of selection pressures on that prey. In terms of *H. bacteriophora*-infected insects, selection by predators (or scavengers) is a major driving force as consumption of infected hosts is fatal for the internally reproducing parasite colony. This selection may therefore explain the diversity of defences we see in infected hosts, such as aposematism, the foul-smell and bioluminescence of infected cadavers.

Vulnerability of infections

Potential hosts for infection however are largely soil-dwelling so there will be little chance for infected hosts to be aposematic against their background if they remain in the soil, although the true enemies of the colony are not known. Red

colouration however is a typical warning colour and often maximises visibility against brown and grey backgrounds (Cott, 1940). In this study however the red colouration of infected insects was quite cryptic against the bark background. However, there was evidence that as infection stage increased, potential discrimination between prey and background also increased due to increasing JND (Just Near Differences) values. Therefore, later infections were reaching the threshold for discrimination by avian predators as infection increased. This suggests that there could be some vulnerability to early infections whereby it was nearly impossible to distinguish between prey and background. This vulnerability at an early stage is supported by studies which show that some defences, especially the chemical defence, have not yet had time to build up and deter predation (Fenton et al., 2011; Gulcu et al., 2012; Jones et al., 2016; Zhou et al., 2002). However, there is evidence that scent can at least negate these effects by deterring predation by beetles across both early and late infection stages (Jones et al., 2016) and scent cannot be ruled out in these field experiments. Other early acting defences in this system also include bioluminescence which could also provide a protective defence to infected hosts early on during infection whilst other defences, and perhaps conspicuousness, build up.

A role for crypsis?

Additionally, crypsis could play a protective role early during infection as although receiving higher attack rates than prey on a conspicuous background, infected prey still received far fewer attacks compared to uninfected prey. Therefore, if predators have a palatable alternative source of prey, crypsis against the background may prevent detection of infected hosts. Furthermore, although

aposematism and crypsis are located at opposite ends of the conspicuous continuum there is evidence that they can combine as a result of distance dependence (Tullberg, Merilaita, & Wiklund, 2005). The idea that individuals are conspicuous at close range and cryptic at a longer distance has been suggested by a number of researchers (Deml & Dettner, 2003; Edmunds, 1974; Endler, 1978; in Ruxton et al., 2004). Tullberg et al., (2005), showed, using human subjects as predators, that cryptic individuals were detected slower than aposematic individuals at a close distance and vice versa at longer distance. This could be important in *H. bacteriophora*-infected individuals as crypsis from a distance could decrease the chance of infected hosts being identified, and if they are, aposematism could be used at a close distance to deter predation. However, this needs further study.

Furthermore, arthropod predators have a large influence on small insect prey and are likely to encounter infected hosts whilst foraging in soil. Insect predators however are limited in their resolution and viewing distance due to the structure of their compound eyes (Land, 2003). Additionally, a large number of insects and spiders lack a dedicated 'red' receptor (Thery & Gomez, 2010). Therefore, reds and oranges, though commonly used in aposematic signalling (Thery & Gomez, 2010), will likely not have as great an effect on arthropod predators. Fabricant & Herberstein, (2015), recently showed that the orange colouration of shieldback stinkbug (*Tectocoris diophthalmus*), although aposematic to birds, is cryptic to mantids. Therefore *H. bacteriophora*-infected individuals may act as aposematic to bird predators but cryptic to insect predators which also encounter infected individuals in the soil substrate.

Host colouration

We ran our first experiment utilising waxworms as predators however the difference between uninfected and infected individuals was quite extreme, changing from white as an uninfected to pink as a day 3 post-infected. We therefore decided to run the experiment with mealworm larva, to see whether host colour had an effect on predation rates. Utilising mealworms on a white background, purely as a comparison we found that predators attacked infected mealworms less than uninfected mealworms. This therefore supports the idea that predation effects seen in both mealworms and waxworms are mostly driven by the presence of infection, rather than uninfected individuals. Additionally, the second experiment also suggests that host colouration is not as important in terms of predator deterrence as a melanised cuticle performed as well as the non-melanised cuticle of the waxworm. This is supported by the idea that *H. bacteriophora* is a generalist and infects a wide range of hosts (Poinar, 1975).

In conclusion, we provide evidence that *H. bacteriophora* infections were attacked and consumed less when conspicuous against their background. Additionally, as colour intensified with infection stage, avoidance did too. However, infected hosts are not likely to be conspicuous against their background as infected hosts are found in the soil substrate where the brown colouration of soil may match the red colouration of infected hosts. Therefore, infected individuals could be acting cryptically at a distance and aposematically in close contact. Furthermore, depending on the visual capabilities of the predators likely to encounter prey, the infected hosts may act cryptically, as in the case of insect predators, or aposematically, in the case of avian predators. Additionally, as conspicuousness was

lower early on during infection, steadily increasing as infection increased, other defences may be prioritised at this time to deter predation. Furthermore, we provide evidence that colouration of the host nematodes infect does not play a major role in predator deterrence as we found similar attack results utilising both waxworms and mealworms as hosts.

2.7 Acknowledgements

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Chapter 3. “Parasite-induced aposematism” protects entomopathogenic nematode parasites against invertebrate enemies

This chapter is published in Behavioural Ecology (Jones *et al.,* 2016. Behavioural Ecology **27**, 645-651) and a copy of the final article is located at the end of this thesis.

3.1 Author contributions

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Andy Fenton and Mike Speed provided comments on the manuscript and discussion of ideas. I designed the study, collected the study organisms and data, conducted the analysis and wrote the manuscript. Mike Speed assisted with the discussion for publication and so the discussion that appears in this chapter is my own interpretation of the results.

3.2 Abstract

Aposematism is a well-known strategy in which prey defend themselves from predation by pairing defences such as toxins, with warning signals that are often visually conspicuous colour patterns. Here we examine the possibility that aposematism can be induced in a host by colonies of infectious parasites in order to protect the parasites from the consequences of attacks on the host. Earlier studies show that avian predators are reluctant to feed on carcasses of host prey that are infected with the entomopathogenic nematode, *Heterorhabditis bacteriophora.* As the age of infection increases, so the parasites kill and preserve the host and subsequently cause its colour to change, becoming bright pink then red. Nematode colonies in dead hosts may also be vulnerable however to nocturnally active foragers that do not use vision in prey detection. Here then we test a novel hypothesis that the nematode parasites also produce a warning odour, which functions to repel nocturnally active predators, (in this case the beetle *Pterostichus madidus*). We show that beetles decrease their feeding on infected insect prey as the age of infection increases; and that olfactory cues associated with the infections are effective mechanisms for deterring beetle predation, even at very early stages of infection. We propose that “parasite-induced aposematism” from the nematodes serves to replace the anti-predator defences of the recently killed host. Because sessile carcasses are exposed to a greater range of predators than the live hosts, several alternative defence mechanisms are required to protect the colony, hence aposematic signals are likely diverse in such “parasite-induced aposematism”.

3.3 Introduction

Parasite-induced alteration of host phenotype is a widespread strategy of transmission among pathogens (Moore, 2002). Many parasites manipulate their host's behaviour or colouration to maximise transmission to a definitive host by making the intermediate host more conspicuous to predators, the definitive host (Moore, 2002). For example, ants infected with the trematode *Dicrocoelium dendriticum* move up to the top of vegetation, increasing their chance of being eaten by grazing sheep, the definitive host (Moore, 1995). Thus, the parasite increases its chance of transmission by increasing the likelihood of the intermediate host being consumed by the definitive host species. However, some parasites only have one host in their life cycle and as a result, predation of this host can be detrimental to the parasite if it is unable to survive and reproduce within the predator. Here we demonstrate a novel form of odour-based host manipulation by a parasitic nematode in order to deter predators from consuming an infected host, protecting the nematode-bacterium colony within.

Entomopathogenic nematodes (EPNs, obligate insect parasites) infect and kill insect hosts. They make use of an obligate bacterial symbiont that first kills the insect host and then suppresses the growth of microbial competitors, preventing the host carcass from decomposition (Waterfield, Ciche, & Clarke, 2009). A well-studied example of this symbiosis is the EPN, *Heterorhabditis bacteriophora* (Nematoda, Rhabditidae) and its symbiotic bacterium, *Photorhabdus luminescens* (Clarke, 2008; Dillman et al., 2012; Waterfield et al., 2009), which infect a large range of soil-dwelling insects. As with other EPNs there is an incubation period between initial infection and release of infectious juvenile forms into the surrounding soil to find new

hosts. For *H. bacteriophora* and *P. luminescens* this incubation period may be as long as 20 days (Clarke, 2008). If foraging animals attack and consume the host carcass during the incubation period they will ingest the entire colony. Ingested nematodes are very unlikely to survive in the predator's gut, and are not known to infect the predator (Fenton, Magoolagan, Kennedy, & Spencer, 2011). Hence ingestion is very likely terminal for the colony. A key, but underexplored, question in understanding the biology of EPNs is then how colonies protect themselves from such a fatal attack by foraging animals during this prolonged period of vulnerability.

Recently Fenton, Magoolagan, Kennedy, & Spencer, (2011) proposed a novel hypothesis that we term "parasite-induced aposematism" as the key strategy in colony defence. In aposematism a chemical defence, such as a toxin, is associated with a warning signal such as a conspicuous colour pattern seen in many toxic species (e.g. ladybirds *Coccinella septempunctata*) or venomous species like many wasps and bees (Mappes, Marples, & Endler, 2005). A conspicuous colour pattern is easier for a predator to detect against a background but it is also easier to learn and remember (Roper, 1990). This effect is then further enhanced by the presence of the chemical defence (Gamberale-Stille & Guilford, 2004; Guilford, 1990; Holen & Svennungsen, 2012; Skelhorn & Rowe, 2006). Fenton et al., (2011) proposed that the nematode and its symbiotic bacterium protect their host's carcass by causing it to manifest aposematic traits.

In support of this "parasite-induced aposematism" hypothesis, colonies of several species of EPN, including *H. bacteriophora* are known to confer chemical defence on host carcasses, repelling species of ant (Baur, Kaya, & Strong, 1998; Gulcu, Hazir, & Kaya, 2012), beetles (Foltan & Puza, 2009), crickets and wasps (Gulcu et al., 2012). Host carcasses infected with *H. bacteriophora* are known to be

protected through repellent metabolic products of its bacterial symbiont (Zhou, Kaya, Heungens, & Goodrich-Blair, 2002; Clarke, 2008). In *P. luminescens,* an insecticidal protein toxin complex is secreted after insect death (toxin complex A, "Tca"), which is known to kill or delay growth of insects, including the Colorado potato beetle, *Leptinotarsa* decemlineata, and the sweet potato fly, *Bemisia* tabaci (Blackburn, Domek, Gelman, & Hu, 2005). Therefore the orally toxic Tca is likely targeted toward foraging scavengers such as ants and other soil-dwelling predators (Daborn, Waterfield, Blight, & Ffrench-Constant, 2001; Waterfield et al., 2009). Hence one component of aposematism, chemical defence, is clearly present in EPNs and its molecular basis is sometimes known.

Fenton et al., (2011) also argued that the second component of aposematism, conspicuous warning colouration is also present in infected carcasses. In *H. bacteriophora* infections there is a transient period of host bioluminescence between 24 and 36 hours after infection, which is conferred by the bacterium (but not in other EPNs which lack *P. luminescens*) (Waterfield et al., 2009). This could conceivably act as an aposematic cue. However in *H. bacteriophora* and commonly in other EPNs there is a longer lasting colour change to the host's epidermis which, in *H. bacteriophora*, goes through orange to bright pink-red after 7 days. This pigment is also produced bacterially (Clarke, 2008). Fenton et al., (2011) demonstrated that naïve European robins (*Erithacus rubecula*) were significantly less likely to handle or consume waxworms (*Galleria mellonella* larvae) that had changed colour after infection by *H. bacteriophora* compared to uninfected individuals.

Though parasite-induced warning colouration seems a likely explanation, it is in our view unlikely to be the whole story of colony defence in EPNs. Warning colouration is, for example, unlikely to protect prey from nocturnally active soil

dwelling predators such as beetles and spiders that have poor vision and operate in low levels of ambient light. Without a warning cue these foragers could cause damage to the carcass and injure the colony within it before being repelled by the chemical defence. Hence we argue that an alternative, nonvisual first line of defence is likely to deter non-visual predators or those foraging at night. When culturing *H. bacteriophora* in the laboratory we noted a pungent odour associated with infections (and not with uninfected, decaying carcasses), and hypothesised that this odour might act as an aposematic cue in itself, repelling and causing wariness in nocturnally active predators (Eisner & Grant, 1981). We investigate whether this olfactory cue can function as an aposematic cue.

A second point of interest is that colony defences are not necessarily produced instantaneously with infection. Rather the epidermal colour changes take several days to develop (e.g. Fenton et al., 2011), and conceivably this may be the case with protective toxins too (see Gulcu et al., 2012). Hence we hypothesised that olfactory aposematism might be in place more rapidly than odour and toxicity changes, providing an early line of defence, while the other components of aposematism build up.

Here then we test this hypothesis of olfactory infectious aposematism with experiments using nocturnal, soil-dwelling beetles (*Pterostichus madidus,* Coleoptera, Carbidae) as predators. We sought to investigate the dynamics of chemical and aposematic defences with *H. bacteriophora* infections, measuring changes in protection associated with changes in phenotypes over time.

We performed two experiments to test these hypotheses: the first examined feeding-related behaviours of a nocturnally active, non-visually hunting forager (the

beetle *Pterostichus madidus*) (Wheater, 1989) in relation to infected or uninfected waxworms; the second the effect of infected or uninfected waxworm odour on the beetles.

3.4 Methods

Beetle collection & housing

Ground beetles (Coleoptera: Carabidae) were trapped in pitfall traps located in a small wooded area at Dale Hall of Residence (University of Liverpool, Mossley Hill, Liverpool). Seven unbaited traps were set up in a transect 1m apart using plastic tumblers with a diameter of 7cm, with a 20x12cm cardboard cover. Trapping ran from 01/07/13 – 05/08/13 and from 19/05/14 – 03/09/14, and ground beetles (henceforth beetles) were collected from traps every three days. Manual foraging, i.e. turning over logs was carried out at Ness Gardens (Neston, Wirral) on 03/07/13. In 2013, 38 *Pterostichus madidus* were caught and in 2014, 62 *P. madidus* were caught. Beetles were sexed after both experiments. Data were pooled across both years since there was no effect of year on time spent feeding (MCMCglmm, p= 0.726), time spent in the circle (MCMCglmm, p=0.634) or time spent on a scent (MCMCglmm, p=0.988). Experimental set up and housing was consistent across both years.

Beetles were housed in individual rectangular containers (Smart Tubz, Tesco, 11cm x 16cm x 4.5cm) with circa 2cm of soil, small twigs (for hiding) and dog food (Cesar's Country Chicken and Vegetable) was provided *ad libitum* as food. Beetles were also sprayed weekly with a hand-operated plant mister and were kept under a

photoperiod of 18:6 L:D at 20 ± 1ºC. Beetles were given seven days to acclimatise to the photoperiod and surroundings before any experiments commenced and allowed a further week between experiments. A total of 53 male and 27 female beetles were utilised in all the experiments and were sexed when dissected following trials (Supplementary material, S1).

3.5 Experiment 1: Effect of nematode-bacterium infection on predation by ground beetles

To test whether nematode-infected carcasses have protection against invertebrate foragers we presented individual beetles with a single waxworm larva in a small behavioural arena and recorded their behaviours in relation to a larva that was either infected or uninfected.

Waxworm infection

Waxworms (*G. mellonella*) were infected with *H. bacteriophora* (strain TTO1 supplied by D. Clarke and S. Joyce from University College Cork) using standard techniques in which ten waxworms were placed on filter paper with 1000IJs/ml of nematode culture in a 90mm petri dish (Kaya & Stock, 1997). Waxworms were then frozen 3, 5 and 7 days post-infection along with fresh uninfected waxworms. Each beetle was used for two trials, one with an infected waxworm of a specified stage of infection and one with an uninfected waxworm. Order of presentation was systematically randomised so that e.g. 15 beetles had an infected waxworm first, whereas 15 received the uninfected waxworm first. We left at least 7 days between

presentations. We aimed for 15 beetles in each subgroup, but deaths of some animals left the subgroups smaller than this (day 3 post-infection trials, infected first presentation =13, uninfected first =13; day 5 post infection, infected first = 15, uninfected first =13; day 7 post infection, infected first =15, uninfected first= 12). Beetles were deprived of food for 24 hours prior to each trial.

The experimental arena was a petri dish in which a target area was marked with a black marker pen (Fig. 3.1a; a part circle, 3cm diameter, centred on a position at the edge of the dish). Beetles were given 10 minutes to habituate to the empty dish, then an infected waxworm (day 3, 5 or 7 post-infection, average weight = 0.249g, sd= 0.016) or an uninfected waxworm larva (average weight= 0.252g, sd= 0.016) was placed in the centre of the target area, and an experimental beetle was moved opposite. There was no significant difference in the weight of infected or uninfected waxworms (W= 3751.5, df= 79, p=0.2008). Beetles were observed for an hour in a dark room, illuminated by a low intensity red light to allow observation of the beetles.

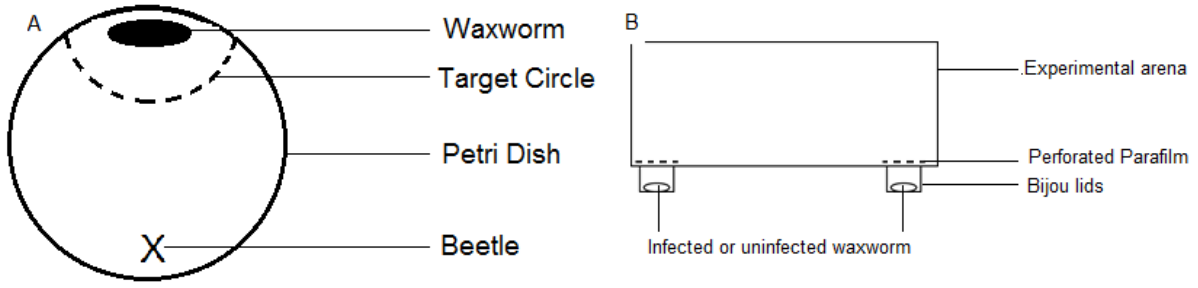

Figure 3.1. Experimental set-up for a) Experiment 1; Petri dish experimental arena with the target area drawn. Infected or uninfected waxworms were placed in the centre of the target circle during trials and beetles were moved to position X at the start of each trial and b) Experiment 2; Lateral view of the scent test arena with fresh infected or uninfected waxworms placed in each bijou lid. Opaque Parafilm™ with pierced holes allowed scent to diffuse but no visual signal.

We recorded the total duration spent in the target area and time spent feeding (mandibles in contact with the waxworm). To see if chemical repellents affected beetle hygiene behaviours, we also recorded the number of antennal cleans and the total time spent on mandibular cleaning with front legs. For time spent in the target area, timing would not start until the main body of the beetle was within the target; legs only were not counted. After the experiment the beetles were fed, weighed one week later and then trialled with the reverse condition (those that received uninfected waxworms first, then received infected waxworms and vice versa) at least one week after the initial trial.

Statistical Analysis

Data were pooled across the two trapping seasons as experiments for different infection stages occurred over both years. Most of the data was left-skewed and conformed reasonably to an exponential distribution and so were analysed using

MCMCglmm in R (Hadfield, 2010). Infection status of waxworms was used as a fixed factor, beetle weight and beetle sex as covariates and order of presentation was included as a random variable, controlling for effects of pseudo replication. The data for the number of antennal cleans were heavily skewed by zero values for day 7 post-infection data, so a Wilcoxon matched-pairs test (with zero values) was utilised, otherwise we used an exponential distribution for days 3 and 5. All MCMCglmm analyses were run for 13000 iterations with a thinning interval of 10 iterations. The feeding data for day 3 and 5 post-infection however were not normally distributed and could not be transformed or the appropriate families found in mixed model programs in R. These data were further analysed using a Mann Whitney test to examine the effect of beetle sex on feeding on uninfected and infected waxworms. These data were therefore analysed using a non-parametric Wilcoxon matched-pairs test. The data for the beetles that did not attempt to feed over the three infection stages were analysed with a binomial glm using day as a fixed factor. When comparing the infected and uninfected waxworm weights for these trials the data were not normal and could not be transformed to normal so a Mann-Whitney test was utilised.

There were only 23 cases of mandibular cleaning across all infection stage experiments and so these data were not analysed.

Experiment 1: Results

There was a significant interaction between prey type and beetle sex on the time spent in the presence of infected or uninfected waxworms (Fig. S3, p=0.004). Female beetles spent less time in the presence of infected and uninfected waxworms than male beetles although the difference was greater when females

were presented with uninfected waxworms. Beetles spent more time in the presence of the uninfected than the infected waxworms (Fig 3.2, MCMCglmm; p<0.001 for all infection stages). Additionally, for beetles receiving infected waxworms 5 days post-infection, there was a prey type x order bias whereby beetles with experience of infected waxworms during their first trial spent more time near uninfected waxworms on their second trial compared to those who had experienced uninfected waxworms on their first trial (MCMCglmm; p<0.001). Comparing time spent near infected waxworms across all three infection stages, there was a marginally non-significant effect in which beetles spent more time in the target circle with 3 day infected waxworms compared to that spent with days 5 and 7 (MCMCglmm; *p*=0.062). This indicates that at day 3 of infection the repellent properties of the infected prey may have been less intense than at later stages of infection.

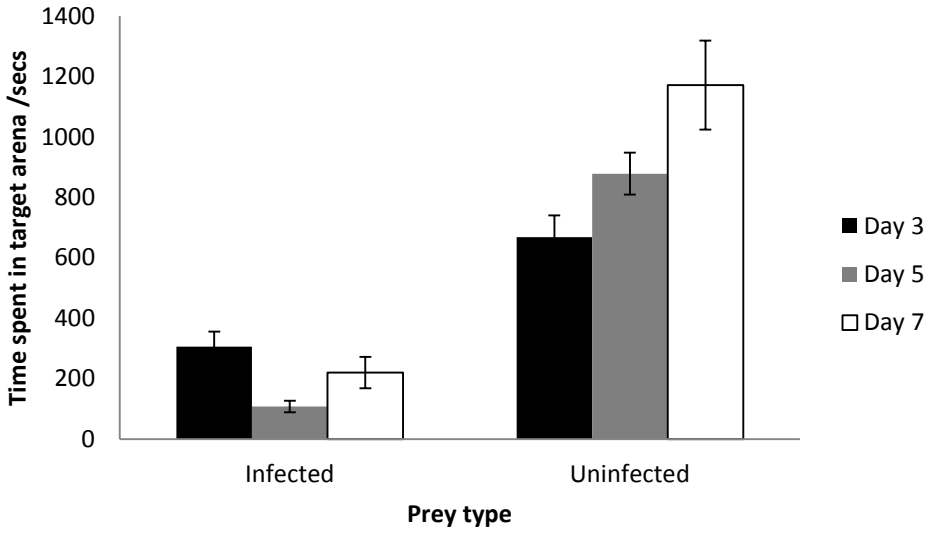

Figure 3.2. Time spent by *Pterostichus madidus* in a target with either day 3, 5 or 7 *H. bacteriophora*-infected or -uninfected waxworms. Data are shown as means ± SE.

Beetles spent significantly more time feeding on uninfected waxworms than infected waxworms at each infection stage (Fig. 3.3; MCMCglmm; day 3; p<0.001,

day 5; p<0.001, day 7; *p*<0.001); there was no effect of order of presentation in this test (MCMCglmm; day 3; p=0.644, day 5; p=0.302, day 7; *p*=0.646) or of sex on day 3 (Mann-Whitney test, uninfected, *p* = 1, infected, *p* = 0.3454) or day 7 (MCMCGlmm, *p*= 0.432). However, female beetles spent less time feeding on uninfected waxworms compared to male beetles at day 5 post-infection only (Mann-Whitney test, *p*=0.01954). However, demonstrating a delay in development of chemical defence, the beetles fed more on day 3 post-infection waxworms than on either day 5 or day 7 post-infection waxworms (MCMCglmm; p=0.040). There was no significant difference in time spent feeding on uninfected waxworms across all three infection stages (MCMCGlmm, *p*=0.614).

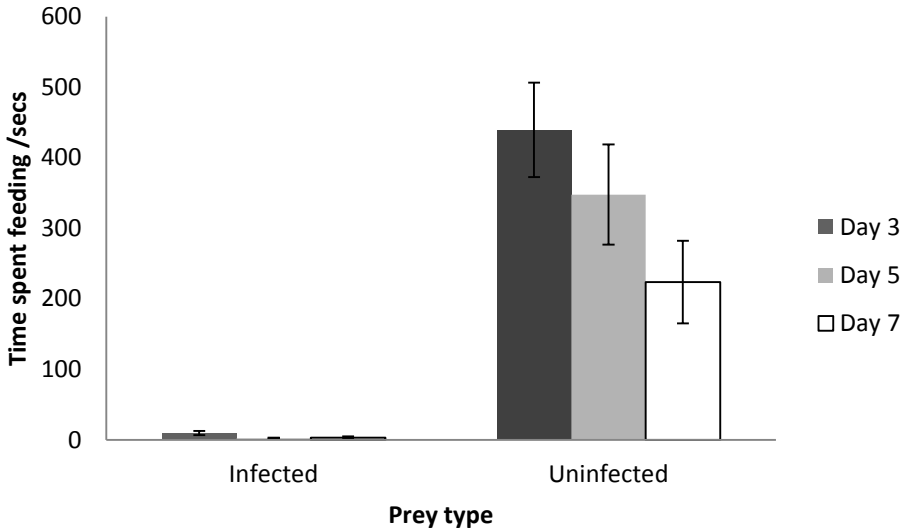

Figure 3.3. Time spent feeding by *Pterostichus madidus* on either day 3, 5 or 7 *H. bacteriophora* infected waxworms or uninfected waxworms. Data are shown as means ± SE.

Similarly, there was a significant difference in the number of feeding attempts on infected waxworms across the three infection stages, with beetles having significantly more feeding attempts on uninfected than infected waxworms at both

days 5 and 7 post-infection (Fig. 3.4., MCMCglmm; day 5; p=0.034, day 7; p<0.001). However, and again supporting the view that early infections have little chemical defence, at day 3 post-infection beetles did not have significantly more feeding attempts on uninfected compared to infected waxworms (Fig. 3.4; p=0.258). There was no effect of sex on the number of feeding attempts (MCMCGlmm, day 3, *p*=0.130, day 5, *p*=0.184, day 7, *p*=0.424).

Given that vision is not a likely cue for the beetles there is evidence that odour itself can protect the carcass from attack. Increasing the age of infection significantly increased the proportion of beetles that did not ever feed on the infected host during the trial (3 days post infection= 38% of beetles; day 5 = 56% of beetles; day 7 = 67% of beetles; binomial GLM: *z*=2.027, df=1, *p*=0.0426). In contrast only 27.5% of beetles never attacked an uninfected waxworms across all infection stages. However as the beetles could examine waxworms with their antenna, we could not rule out that some of this avoidance was due to direct chemical assessment, and some due to olfaction. Hence in the next experiment we tested the role of olfaction specifically.

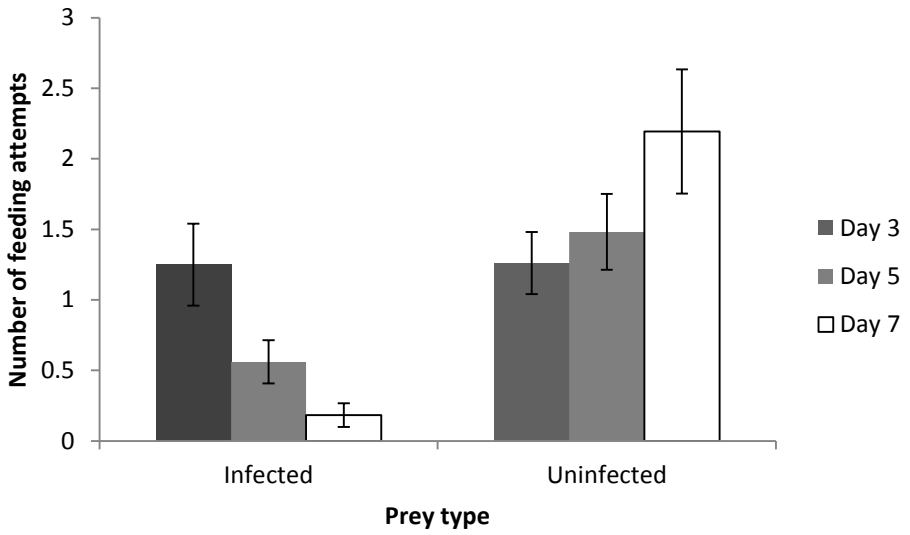

Figure 3.4. Number of feeding attempts made by *P. madidus* on either day 3, 5 or 7 post-infection *H. bacteriophora*-infected and -uninfected waxworms. Data are shown as means ± SE.

Finally in this experiment there was no significant difference between the number of antennal cleans performed by *P. madidus* upon encountering infected or uninfected waxworms (Fig. S2 (supplementary materials), MCMCGlmm $p>0.05$ for days 3, 5 and 7 for both the number of antennal cleans (day 3; $p=0.750$, day 5; $p=0.734$, day 7; $p=0.852$ and antennal cleans *per se* (present or absent) (day 3; $p=0.639$, day 5; $p=0.714$, day 7; $p=0.208$). Furthermore, there was no effect of beetle sex on the number of antennal cleans performed. However, there was a significant negative effect of beetle weight on the number of antennal cleans performed when beetles were exposed to day 7 post-infection either infected or uninfected waxworms ($F_{1,26}=4.609$, $p=0.041$), so that bigger beetles made fewer cleans than smaller beetles.

There were only six episodes of mandibular cleaning (beetles utilising their front tarsi to 'wipe' their mandibles) during the day 3 post-infection experiments, 13

during the day 5 post-infection experiments and four during the day 7 post-infection experiments. The time spent mandibular cleaning ranged from 2-83 seconds and the majority of episodes were observed in *P. madidus* that were trialled with infected waxworms.

3.6 Experiment 2: Is there olfactory protection of infected waxworms?

This experiment was designed as a two-choice preference test (Fig. 3.1b). Scent test arenas were created using plastic food containers (Smart Tubz, Tesco, 11cm x 16cm x 4.5cm) with two bijou bottle lids (diameter=15mm, height=10mm) as scent wells positioned 12 cm apart (See Figure 3.1b). Square pieces of opaque Parafilm™ were then used to cover the scent wells, and 21 holes were pierced with a needle in a grid-like fashion for aeration.

To provide scent cues, 0.3g of macerated fresh infected (either days 3, 5 or 7 post-infection) or fresh uninfected waxworms were measured and put into opposite lids. During an experimental trial beetles were observed for one hour and we recorded the time spent in proximity to each scent well. Beetles were tested in two trials with the position of the infected waxworm reversed between them (with a minimum of 7 days between first and second trials). Hence approximately half the beetles (n=10) received scent from uninfected waxworms on the right hand side and the others (n=9) received scent from uninfected waxworms on the left hand side. Arenas were re-used between trials, but were cleaned with 70% ethanol to prevent beetles leaving olfactory cues to other subjects. Fresh olfactory cues were made on each day of the experiment.

We used the same set of beetles as in experiment 1, 10 days after the final trial of that experiment, therefore beetles were experienced predators. As before beetles were starved for 24 hours prior to experimentation. Four beetles died after one trial, with exposure to both infected and uninfected scent, and so were removed from the experiment and five died before the experiment started. We again used MCMCglmm (Hadfield, 2010) in R, for an exponential distribution. Infection status of waxworms was used as a fixed factor, beetle weight and sex as covariates and order of presentation was included as a random variable, controlling for effects of pseudo replication. Data were pooled across both years as olfactory experiments for different infection stages were run across the two years.

Experiment 2: Results

In general, beetles avoided the scent of *H. bacteriophora*-infected waxworms. They spent significantly more time on the uninfected than infected scent across all infection stages (Fig. 3.5, MCMCglmm; day 3; $p=0.012$, day 7; $p<0.001$). There was no effect of beetle mass, sex or order of presentation (i.e. left or right side bias) in either the day 3 or day 7 test. For the day 5 post-infection scent test there was a side x prey type bias (MCMCglmm; $p=0.034$) which showed that beetles spent more time feeding on uninfected waxworms when the infected scent was located on the left hand side of the experimental arena. There was no effect of sex in the day 5 test.

Notably there was no significant difference in time spent on the infected scent across all three infection stages (MCMCglmm; $p=0.448$). Therefore beetles showed similar avoidance of the scents of day 3, 5 and 7 post-infection waxworms.

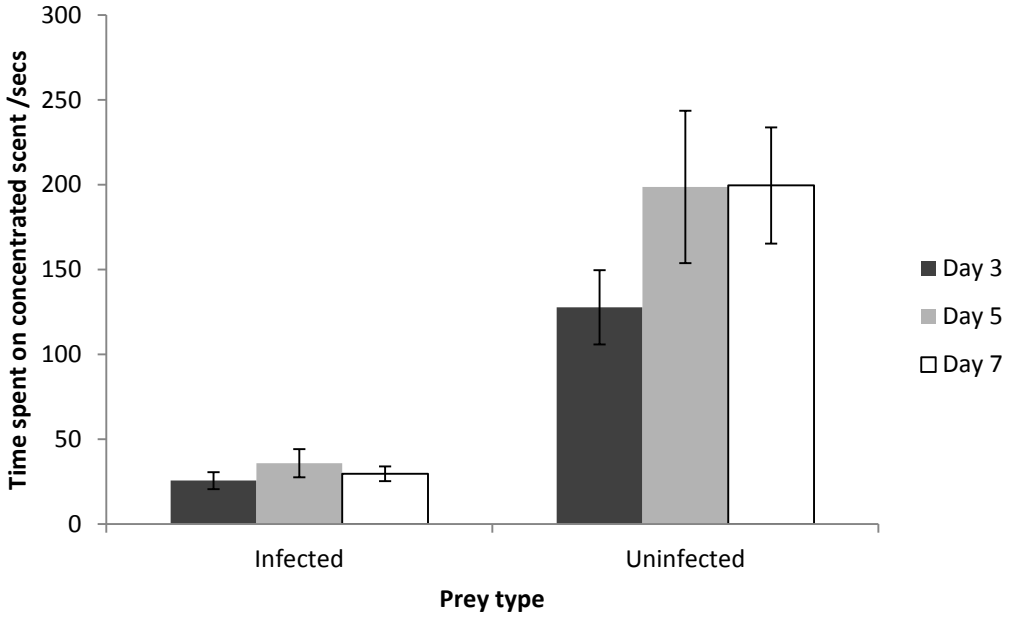

Figure 3.5. Time spent on either a day 3, 5 or 7 *H. bacteriophora*-infected waxworm or -uninfected waxworm scent by *P. madidus*. Data are shown as means ± SE.

3.7 Discussion

We provide evidence of a novel olfactory deterrent in this nematode-bacterium system which builds on other studies highlighting a visual and chemical deterrent (Baur *et al.,* 1998; Zhou *et al.,* 2002; Gulcu *et al.,* 2012, Fenton et al., 2012). Furthermore, this olfactory deterrent protected all stages of infection, before other defences could build up. This suggests that the protective olfactory signal may act as a preliminary barrier to predation whilst other defences build up.

We found evidence of a chemical deterrent (experiment 1) which supports other studies examining chemical defence in this system ((Baur et al., 1998; Gulcu et al., 2012; Zhou et al., 2002). Beetles approached and attacked fewer infected waxworms compared to uninfected waxworms. However, we found that infected waxworms were more vulnerable early on (day 3) compared to later infections (day 5 and 7) as beetles spent more time feeding and attempting to feed on day 3 infections. This is consistent with foraging preferences of wild birds (Fenton et al., 2011) and ants, crickets and wasps (Gulcu et al., 2012) that also showed less of an aversion to early stage infections. Additionally, female beetles spent less time than males near infected and uninfected waxworms and feeding on uninfected waxworms, although this may be due to females being less active during summer following egg laying (Matalin, 2008).

We have therefore been able to demonstrate (for the first time to our knowledge) that an olfactory cue can protect hosts infected with EPNs across multiple infection stages. Olfaction may therefore work as a preliminary defence against potential predators, specifically nocturnally foraging invertebrate predators. This may then be supported later on during infection as the chemical defence builds up to suitable levels to deter predation. This olfactory cue however is not just as a result of decaying individuals as infected hosts don't decay during infection, actually remaining turgid due to preservation of the host through antimicrobials synthesised by *P. luminescens* (Clarke, 2008). Therefore, this olfactory cue is conferred by the EPN and/or its symbiont and could play a major role in the protection of infected hosts from potential predators.

Olfactory aposematism

During our olfaction experiment (experiment 2), we found that beetles spent a small amount of time next to infected hosts, regardless of infection stage, similar to the first experiment. Therefore, with a potentially palatable alternative source of prey, beetles utilise the olfactory cue to avoid infected hosts and orientate towards the uninfected prey. This is highly beneficial to the host as it minimises risk of predation and death for the parasitic colony inside as the chemical deterrent builds up. Eisner at al. (1981), suggested that olfactory aposematism may be important in warning off predators. This has been seen in plants, which advertise their unpalatability through the use of warning odours (Camazine, 1985). Additionally, there is a wealth of evidence of olfactory aposematism in aposematic literature whereby warning odours, usually pyrazine (a common insect warning signal), deter predators from consuming novel or warningly coloured food (Lindstrom et al., 2001; Guilford, 1987; Rowe, 1996; Siddall, 2011). Most of these studies have utilised chicks in a lab environment showing that the presence of a warning odour increases the latency to attack aposematic individuals (Rowe, 1996). Olfactory aposematism therefore is regarded as having the potential to deter predation, as seen in this study.

Vulnerability of early infections

Our results show that new infections (up to day 3) are vulnerable to beetle foragers which supports other results highlighting this fact as well, based on the chemical defence alone. It is therefore interesting to consider why there is a lag in the build-up of defences to protect the host when the earlier they came into effect, the higher the level of protection for infected hosts, hence the lower the risk of predation and death. Infected individuals however undergo a number of changes in

their phenotype; turning red, becoming bioluminescent transiently, producing a foul smelling odour and a chemical deterrent. Investment in each of these defences is likely to be costly and therefore it may take time for each of the defences to build up based on their resource allocation. It is currently unknown how the symbiont invests in each of these defences, whether there's equal investment or one is prioritised over another. However, it seems that the cost of investing in an olfactory deterrent may be less than that of the chemical or visual deterrent, meaning that a relatively cheap defence can be produced during this period of vulnerability during infection to protect the parasite colony whilst more costly defences build up.

Prior to day 3 though, the infected host is still vulnerable to predation but there may be benefits from other host changes that have not yet been considered. As mentioned previously, infected hosts also bioluminesce transiently (Waterfield et al., 2009) shortly after death which could also operate aposematically. Wild toads (*Bufo bufo*), for example, have been shown to lower attack rates and increased latencies towards bioluminescent glow-worm larva (Coleoptera: Lampyridae) in their native range (De Cock & Matthysen, 1999, 2003). It is therefore feasible that bioluminescence in this nematode-bacterium system may confer some protection to the host before build-up of other chemical defences. Due to poor vision in beetles and other invertebrates it seems unlikely that these predators are targeted by this defence. It seems more likely that bioluminescence is likely to target small mammalian predators likely to encounter infected hosts whilst foraging.

Evolution of infectious aposematism

There is good evidence that EPNs protect themselves utilising what we term 'infectious aposematism'. Chemical defence builds up slowly, reaching a peak at day

5 whilst the olfactory defence protects infected hosts throughout infection. Due to poor vision in invertebrate predators, it seems unlikely that the visual defence offers much protection against predation. It is therefore interesting to consider why so many defences exist in this system where perhaps investment in one generally acting defence could be less costly and decrease predation across all infection stages.

Three aposematic signals may therefore exist in this system which may act to deter a range of potential predators: bioluminescence to nocturnal, visually capable mammals (De Cock & Matthysen, 2003); olfaction to nocturnal (potentially diurnal) foragers including visually limited invertebrates; visual to diurnally foraging animals such as birds (Fenton et al., 2011). This 'multimodal' nature of defence in EPNs may therefore be targeted towards predators with different perceptual capabilities (Rowe & Halpin, 2013).

Conclusions

Our work supports the hypothesis of Fenton et al., 2011, that EPNs use 'infectious aposematism' to protect their infected host from predation. Furthermore, we provide evidence that as well as a chemical defence advertised through a warning display, EPNs also utilise olfactory cues to deter predation. This olfactory cue may also target predators who do not attend to the other defences due to their perceptual capabilities, such as nocturnal arthropods. Olfaction may also provide an early defence whilst other defences build up to protect early stage infections.

3.8 Acknowledgements

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3.10 Supplementary material

Table S1. Number of male and female beetles utilised during experiment 1. Beetles were sexed via dissection following the trials.

Day of Experiment	Number of Males	Number of Females
3	17	9
5	14	13
7	22	5

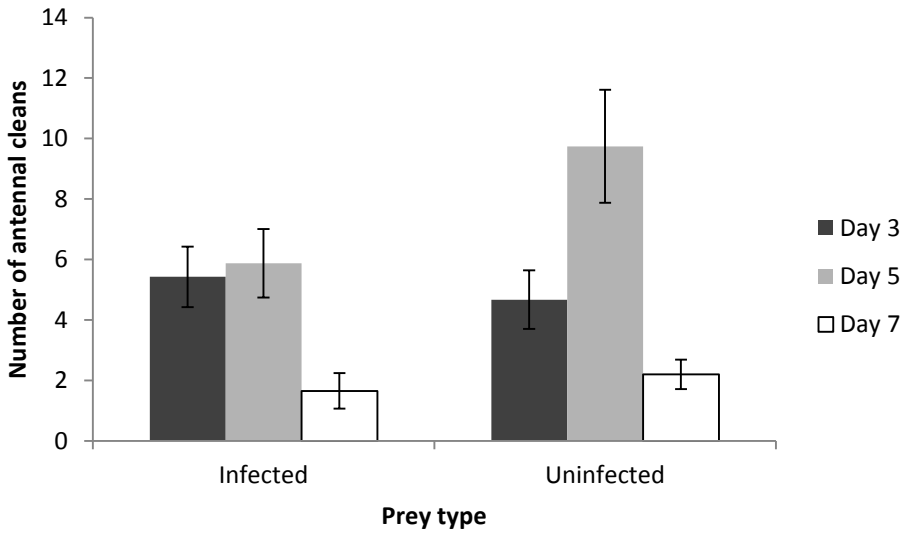

Figure S2. Number of antennal cleans performed by *P. madidus* when encountering either day 3, 5 or 7 *H. bacteriophora*-infected or -uninfected waxworms. Data are shown as means ± SE.

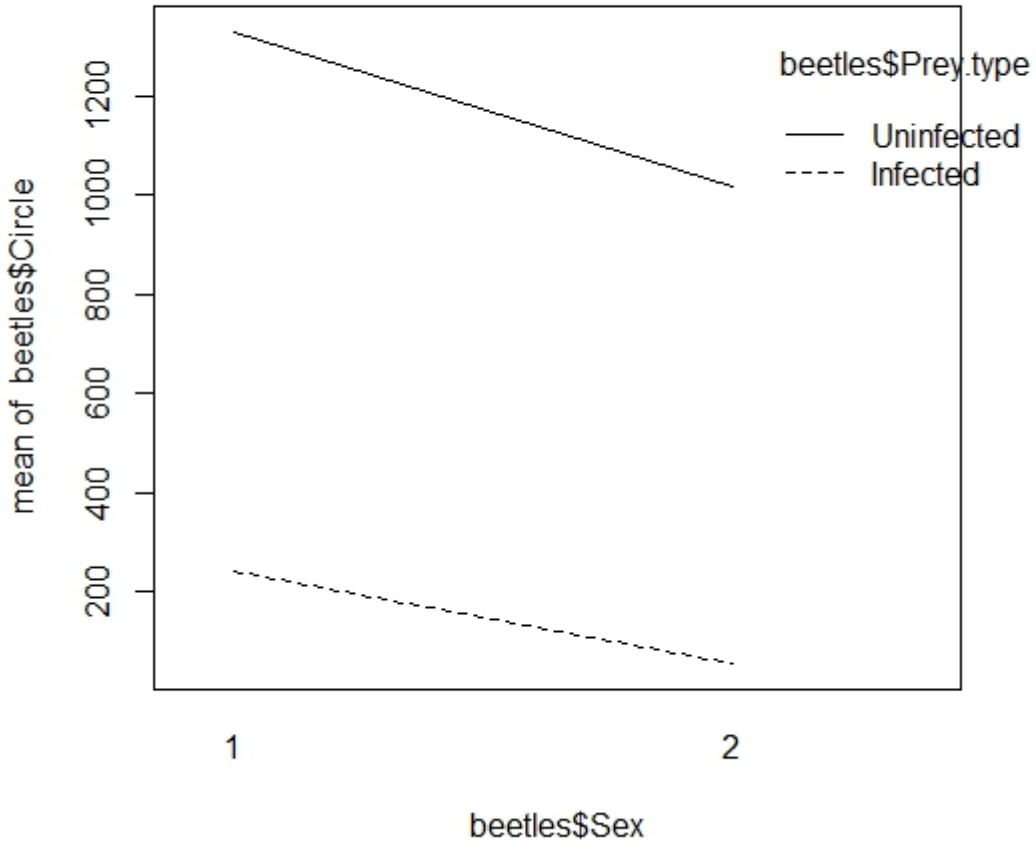

Figure S3. Interaction plot between beetle sex, prey type and time spent in the circle.

Chapter 4. Investment in multiple defences protects a nematode-bacterium symbiosis from predation

This chapter has been accepted for publication in Animal Behaviour.

4. 1 Author contributions

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Andy Fenton and Mike Speed provided comments on the manuscript and discussion of ideas. Johanna Mappes helped with experimental design, analysis and provided comments on the manuscript.

4.2 Abstract

The act of predation often comprises multiple sequential steps in which prey can employ defences at all or some of these stages to deter predation. However, investment in defences is costly unless they are outweighed by conferring some benefit to the bearer. One system that employs multiple defences is that of the entomopathogenic nematode *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*. This nematode-bacterium complex infects and kills soil-dwelling insect larva, in which they then reproduce and juveniles emerge 2 weeks later. Predation of the infected host cadaver at any point during infection is fatal for the parasitic colony inside. Infected individuals however turn red, produce a chemical defence, bioluminesce and smell strongly at various stages of the infection process. We tested whether these colour and scent signals conferred a benefit to the infecting nematode-bacterium complex, utilising feeding trials of nematode-infected waxworms (*Galleria mellonella*) with wild caught great tits (*Parus major*). We found that scent overshadowed colour at various stages of infection, in terms of reducing levels of attack, but not when both signals were in concert in terms of consumption of infected individuals. However, we tested for multimodality, as both signals are in different sensory modalities, and found no overall benefit in terms of initial attack on the first prey item, although this does not rule out the possibility of multimodality within this system.

4.3 Introduction

Predation is virtually ubiquitous in the natural world with many animals experiencing the risk of predation at some part of their life history. This has driven the evolution of a wide variety of anti-predatory defences employed between species (Caro, 2005) and within species (Van Buskirk, 2001). One reason for this is that individuals face attack from different predators, for example plants face attack from multiple predators in the form of insects and pathogens (Maleck & Dietrich, 1999). However, this is not the sole reason for within-individual variation in defences as a single individual can also utilise different defences against different predators in different attacks (Caro, 2005).

The predation process is often broken down into sequential steps with the most frequently cited being those described by Endler (1986, 1991). He proposes that predation can be split into discrete stages consisting of detection, identification, approach, subjugation and consumption (Endler, 1986). Prey are able to counteract this through multiple defences which can act at one or a number of stages, meaning that prey can employ defences at each stage of attack to deter predation. However, defences are usually costly and each additional defence adds an associated cost (Caro, 2005). Different costs of various defences have been considered in depth in Ruxton et al. (2004). Endler (1991) argued that investment in a defence at a given stage of predation would reduce the benefit of investment in later stages, suggesting investment should be biased towards earlier defences. However, there are plenty of examples where individuals do invest in defences in later stages of predation (Edmunds, 1974; Eisner, Eisner, & Siegler, 2005 and references within).

A growing body of literature aims to examine this phenomenon whereby individuals invest in later defences and how prey invests across different defences. Broom, Higginson, & Ruxton (2010) utilised a simple model to explore when prey should invest in a single or multiple defences. When the ratio of the constitutive cost to the benefit of defences is low and similar, the authors predict investment cross both defences. Furthermore, investment in multiple defences at different stages of predation are predicted when defences are relatively cheap or the individual has more resources available for investment in defence (Speed, 2016, in prep.). Additionally, investment in multiple defences has implications for evolution of both predator and prey as successful attack of a predator on prey depends on the number of traits for each species (Gilman, Nuismer, & Jhwueng, 2012).

Although a number of studies have examined multiple defences (Jongepier, Kleeberg, Job, & Foitzik, 2014; Van Buskirk, 2001), multiple defences are normally considered in the context of multiple predators (Maleck & Dietrich, 1999; Poitrineau, Brown, & Hochberg, 2003; Rigby & Jokela, 2000; Sih et al., 1998; War et al., 2012). Individuals are normally attacked by multiple species of predator at some stage of their life cycle and so having multiple barriers, or barriers acting at different stages of predation, would be beneficial. This is supported by literature concerning multimodality where it is suggested that the evolution of multimodal signals may have arisen to target predators with different perceptual capabilities (Rowe & Halpin, 2013). However, what seems to be lacking in this area is the view of multiple defences in a multimodal context. It seems logical that having multiple defences in a sequential fashion is beneficial against a single predator (Chen, 2008 and references within) but they can also be beneficial against a range of predators or parasites (Gilman et al., 2012; Poitrineau et al., 2003; War et al., 2012).

One such system that incorporates both these ideas is that of the entomopathogenic nematode *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*. The nematode infects and kills soil-dwelling larval insect hosts within 48 hours, though rather than decaying (Milstead, 1979), they undergo a number of changes. Once the host is dead, the symbiotic bacteria must provide defences to replace those of the now-dead host (Jones, Fenton, & Speed, 2016). Infected hosts bioluminesce (transiently), turn permanently red, become unpalatable (Ffrench-Constant & Bowen, 2000) and produce a strong-smelling odour. A key point here is that the infected carcass does not decay during the infection, rather it is preserved by antimicrobials synthesised by *P. luminescens* (Clarke, 2008). Hence the repellent odour is not that of a decaying corpse, rather it is something conferred by the EPN and/or its symbiont. Nematodes reproduce within this changing host and emerge 10-14 days post-infection before repeating the cycle of infecting a new host by cruising through the soil (Johnigk & Ehlers, 1999). Hence, predation at any stage will result in nematode and bacterial death. Although each of these defences is a constitutive rather than an induced defence, they occur at different points of infection and at different stages of predation. Following Endler's (1991) framework these various defences mostly fall into the identification stage of predation, with noxiousness in the subjugation stage.

Previous work examining this system has shown an adaptive value to these host changes as the chemical deterrence induced by the symbiotic bacteria deters ants from feeding on waxworms infected with *P. luminescens* (Baur, Kaya, & Strong, 1998; Gulcu, Hazir, & Kaya, 2012; Zhou, Kaya, & Goodrich-Blair, 2002). Furthermore, avian predators also showed an aversion to *H. bacteriophora*-infected waxworms (Fenton, Magoolagan, Kennedy, & Spencer, 2011). This aversion was

primarily attributed to the visual appearance of the infected waxworms. However, this experiment did not explicitly test the olfactory component of this avoidance but, if handled, infected prey tended to be rejected more frequently than uninfected (Fenton et al., 2011). This effect was only seen in prey 5 or 7 days post-infection whereas at day 3 post-infection avian predators were equally likely to select an infected or uninfected waxworm. Furthermore, Foltan & Puza, (2009) found that a related nematode species, *Steinernema affine*, caused beetle deterrence when infected in waxworms. Jones, Fenton, & Speed (2015) have recently reported an olfactory and chemical deterrent towards carabid predators whereby ground beetles avoided the scent of *H. bacteriophora*-infected waxworms across a range of infection stages. However, ground beetles fed on infected and uninfected waxworms to a similar extent during early infection stages, before avoiding infected individuals as infection progressed. Recently, Jones et al., (in prep.) have found that bioluminescence acts as a deterrent early on during infection with house mice (*Mus musculus domesticus*) avoiding bioluminescent over non-bioluminescent prey.

Although deterrent effects have been found for the defences individually (Baur et al., 1998; Fenton et al., 2011; Gulcu et al., 2012; Jones et al., 2016) there have been no studies explicitly testing combinations of these defences to determine why so many barriers to predation exist in this system. Our aim was to test a combination of the olfactory and visual deterrent (both deterrents considered at the identification stage of predation) to determine whether there is an advantage of having either of these defences singly or in concert. To do this we conducted three experiments; the first two to examine the effect of scent and colour in isolation and the third to examine colour and scent in concert. We found differing levels of avoidance of nematode-infected waxworms when cues were presented alone and in concert,

suggesting a benefit to multiple levels of defence within this system. We discuss the results in terms of the evolution of multiple layers of defence and multimodal signalling within a novel aposematic nematode-bacterium signalling system.

4.4 Methods

Experiments were run at the Konnevesi Research Station, University of Jyväskylä, Central Finland from January-March 2014. Permits for experiments with wild birds were issued by the Central Finland Centre for Economic Development, Transport and Environment (KESELY/1017/07.01/2010) and the National Animal Experiment Board (ESAVI-2010-087517Ym-23).

Nematode culturing

Waxworm larvae (*Galleria mellonella*, Livefoods Direct™) were infected with the nematode strain *Heterorhabditis bacteriophora* TT01 (supplied by D. Clarke & S. Joyce, UCC) by infecting 10 waxworms per petri dish containing 90mm filter paper with 1000IJ/ml stock nematode solution. These were then frozen or utilised fresh depending on each of the three experiments.

Bird housing

Ninety wild Great Tits (*Parus major*) were trapped at feeding sites at Konnevesi research station and ringed. Birds were kept in individually illuminated, ventilated plywood cages (64x46x77cm) indoors in a daily light period of 11h 30mins. Sunflower seeds, feed balls and fresh water were available ad libitum except 2 hours prior to trials when birds were food deprived to ensure motivation to forage during

experimentation. All birds were released at their capture sites at the end of the experiment.

Experimental Arena

The experiments were run in illuminated, ventilated plywood cages (50x50x57cm) that contained a perch and fresh water bowl. Birds were allowed to habituate to the experimental cage for at least an hour during which they had to consume two sunflower seeds before the experiments took place. The birds were observed through a one-way plastic front and in a dark room so the birds were less aware of an observer. Due to lack of birds towards the end of the season, some birds (*N*=7) were utilised for multiple trials, however, only across the colour only and scent only trials. Those that experienced colour only had not encountered the smell and vice versa so only these birds were utilised for the second (opposite) trial.

Experiments were run to determine how predators respond to visual and olfactory cues when they are able to feed on prey. However, as predators were not able to feed during the colour only trial, this experiment was used, alongside the others, to test the multimodality of the visual and olfactory signal. We present our results in terms of the attack data, consumption data (except the colour only trial) and then the multimodal nature of the signal.

In all three experiments described below, the birds were presented with two sets of 4 prey in or on petri dishes depending on the experiment. One set were at one of days 3, 5 or 7 post-infection; the other set were uninfected and were killed by freezing on the day of the trials. Fresh uninfected waxworms were utilised during trials although we must consider the effect of age of the cadaver as uninfected individuals will have shown no effects of decay, compared to infected individuals.

Fenton et al., (2011), however showed that wild robins were significantly less likely to attack and consume *H. bacteriophora*-infected waxworms compared to controls, regardless of the age of cadaver of the uninfected controls (i.e. either fresh or decayed for the same amount of time as infected waxworms). Therefore, although we can only interpret our results in the light of freshly killed uninfected controls, we are confident that our results are representative of what would happen with decayed uninfected controls also.

For each experiment, four of each prey type (infected or uninfected) were utilised as birds were seen to attack 8 prey items in total during pilot studies, meaning they could potentially attack all prey items during trials if there was no avoidance of either prey type. Waxworms were weighed beforehand to control for weight across infected and uninfected prey. We varied the stimuli available to the birds between experiments so that there were three conditions (1) scent only, (2) colour only and (3) scent and colour. Thirty birds were utilised per experiment, ten per infection stage for each condition. Following an experimental trial, birds were provided with sunflower seeds *ad libitum* until returned to their home cage.

Condition 1: Scent only

Here we placed four prey (uninfected versus either a day 3, 5 or 7 post-infected waxworm) under one of two obscuring but permeable membrane (odourless triangular bandage) so that the odour but not the colour could be perceived. We placed dead uninfected waxworms on the top of both petri-dishes. Here then the visual stimulus is the same, but the odours (infected vs uninfected) can differ. To maximise concentration of olfactants, the petri dish was sealed with the lid and left for one hour to allow the scent from both fresh infected and uninfected waxworms to

diffuse through the bandage. At the start of the trials, the lid was lifted to allow olfactants to escape.

Of the 30 birds utilised in the trial, half received infected waxworms on their left (Female=6, Male=9) and half received infected on the right (Female=7, Male=8). The birds were observed for 20 minutes after the first attack on either prey and the order of prey taken; number of prey consumed and any rejection behaviour i.e. throwing or dropping of the prey item, was recorded.

Condition 2: Colour only

Four infected and uninfected waxworms were frozen and placed on two layers on odourless triangular bandage underneath the lid of a petri dish to seal the waxworms and stop any olfactory signal. Half the birds received infected prey on the left (Female=5, Male=10), half on the right (Female=6, Male=9). Birds were observed for 20 minutes following an attack on either waxworms and attacks were counted as pecks on the petri dish lid and approaches were counted as lands on the dish.

Condition 3: Colour and Scent in concert

Four waxworms, uninfected or infected, were presented in petri dishes on a couple of layers of odourless triangular bandage, to ensure the same background for all prey during the three experiments. To mirror the scent only condition, the petri dish was sealed with the lid and left for one hour to allow the scent from both fresh infected and uninfected waxworms to diffuse. Birds were then observed for 20 minutes after initial attack for the number of waxworms attacked, consumed and rejected, as well as approaches to each dish. Of the 30 birds utilised in the trial, half

received infected on the left (Female=7, Male=8) and half received infected on the right (Female=6, Male=9).

Statistical Analysis

Rather than analysing each experiment separately, we pooled the data into one model and examined attack rate, consumption rate and multimodality across the three conditions.

Attack rate

We coded whether a prey was amongst the first four (50%) attacked and then how many of these were uninfected or infected prey, then ran a binomial GLM (Generalised linear model) using the package lme4 in R (R Core Team, 2013) examining infection status (either infected or uninfected) and experiment (colour and scent, scent only or colour only) as main effects, for each infection stage separately. For the binomial GLM we utilised the colour and scent in concert experiment as the reference level, with comparisons towards this condition. Here, as well as in other analyses, bird ID was included as a random effect as some birds were utilised in two trials, although this swiftly disappeared from the final model. We used the predict() function in R to plot the means and standard errors for the data.

Consumption rate

We could only examine consumption rate for the colour and scent in concert and scent only conditions so we coded waxworms here as consumed or not. We examined infected prey only as we were interested in parasite colony survival in

these individuals. We firstly examined probability of consumption *per* se and then examined the probability of consumption given an attack had taken place.

Similarly to the attack rate, we examined consumption *per se* for infected individuals for each infection stage separately. We then ran a binomial GLM examining each infection stage in turn with infection (either infected or uninfected) and condition (scent and colour, scent only or colour only), as well as their interactions, as explanatory variables.

Additionally, we examined consumption rates on infected waxworms based on infected prey that were attacked. The data therefore only consisted of binomial data for those infected prey that had been attacked (i.e. where attack=1). We then ran the same GLM as used for the general consumption data, but for infected waxworms consumed given attack.

Multimodality

To examine the benefit of colour and scent signals in concert, we examined the first prey attacked (either infected or uninfected) in every trial for each condition when the birds were naïve. We used Fisher's exact test to analyse a 3 x 2 contingency table (Infection stage x Infection) for the colour and scent, scent only and colour only conditions. We then examined each infection stage in each trial using a chi-square to examine the difference in the numbers of infected and uninfected waxworms attacked. We hypothesised that each signal would have an additive effect on avoidance with the sum of both signals in concert greater than either signal alone.

We also utilised a 9 x 2 contingency table (Infection stage per experiment x Infection) to examine whether there were any differences in the first waxworm attacked across each infection stage across each condition.

4.5 Results

We discuss the results in three ways: firstly, attack rates on infected and uninfected prey across all three conditions; secondly, consumption of infected individuals during the scent only and colour and scent conditions and thirdly, as a multimodal signal.

Attack rate

For the first 4 prey attacked, compared to the colour and scent condition, there was no significant difference in prey attacked based on condition (Fig. 4.1; Scent only: z_{240}=-0.897, *p*=0.3695 and Colour only: z_{240}=0.230, *P*=0.8185) or presence of infection (Fig. 4.1; z_{360}=-1.78, *P*=0.0756) for day 3 post-infection prey. Therefore, infected prey were attacked at similar rates to uninfected prey and did not benefit from having one or two signal components (colour and/or scent).

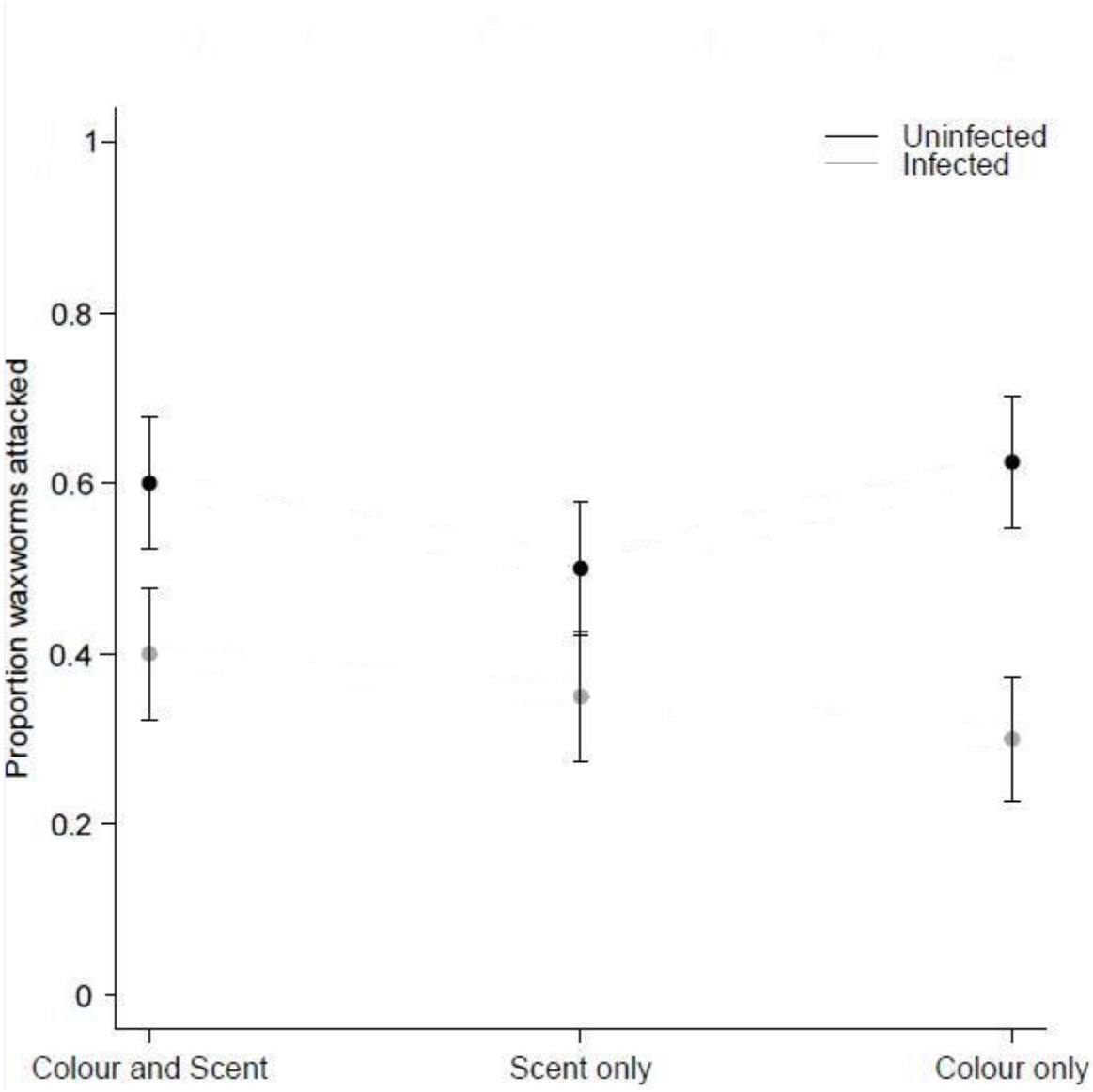

Figure 4.1. Proportion of uninfected or *H. bacteriophora*-infected day 3 post-infection waxworms attacked in the first 4 attacks across the three conditions (Colour and Scent, Scent only and Colour only). Error bars represent 95% confidence intervals.

However, for day 5 post-infection prey there was a significant difference between the attack rates in the colour and scent versus the colour only condition (Fig. 4.2; z_{120}=-2.426, P=0.0153) but not versus the scent only condition (Fig. 4.2; z_{120}=-1.350, P=0.177) and whether prey were infected or not (Fig. 4.2; z_{360}=-5.712,

P<0.001). Therefore, at this stage (5 days post-infection), scent only is as effective a signal to deter attacks on infected individuals as colour and scent in concert. Additionally, there was a two way interaction between scent only and presence of infection (z_{120}=2.178, *P*=0.0294) and colour only and presence of infection (z_{120}=3.360, P=0.0008).

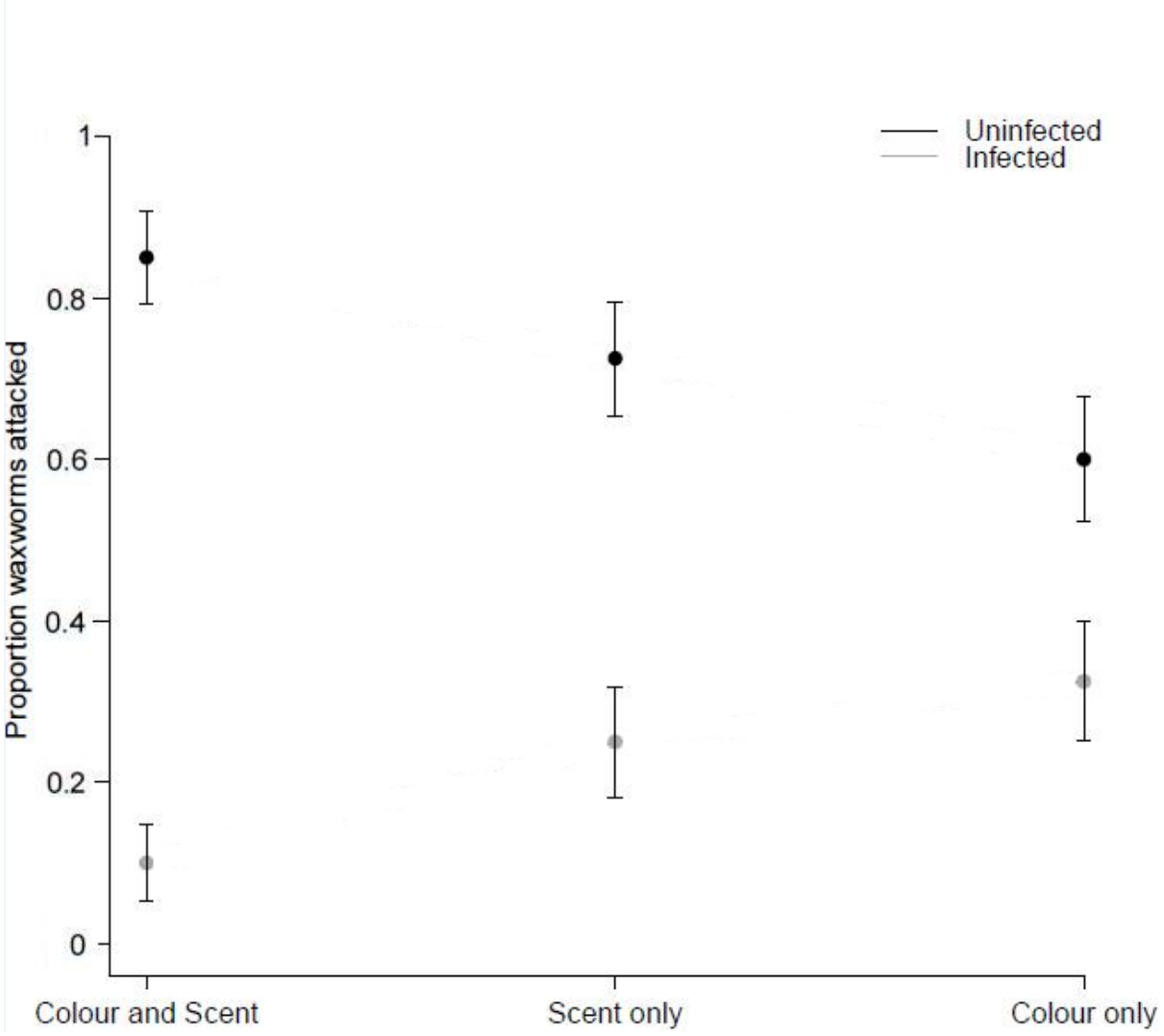

Figure 4.2. Proportion of uninfected or *H. bacteriophora*-infected day 5 post-infection waxworms attacked in the first 4 attacks across the three conditions (Colour and Scent, Scent only and Colour only). Error bars represent 95% confidence intervals.

For day 7, there was a significant difference in attack rate between colour and scent versus scent only (Fig. 4.3; z_{240}=2.012, *P*=0.0442) and whether prey were infected or not (Fig. 4.3; z_{360}=-4.618, *P*<0.001). Additionally, there was an interaction between scent only and infection (z_{120}=-2.581, P=0.0044). Therefore, at this stage, scent only provides the best protection in terms of reduced attacks on infected individuals, but colour only is as protective as both signals together.

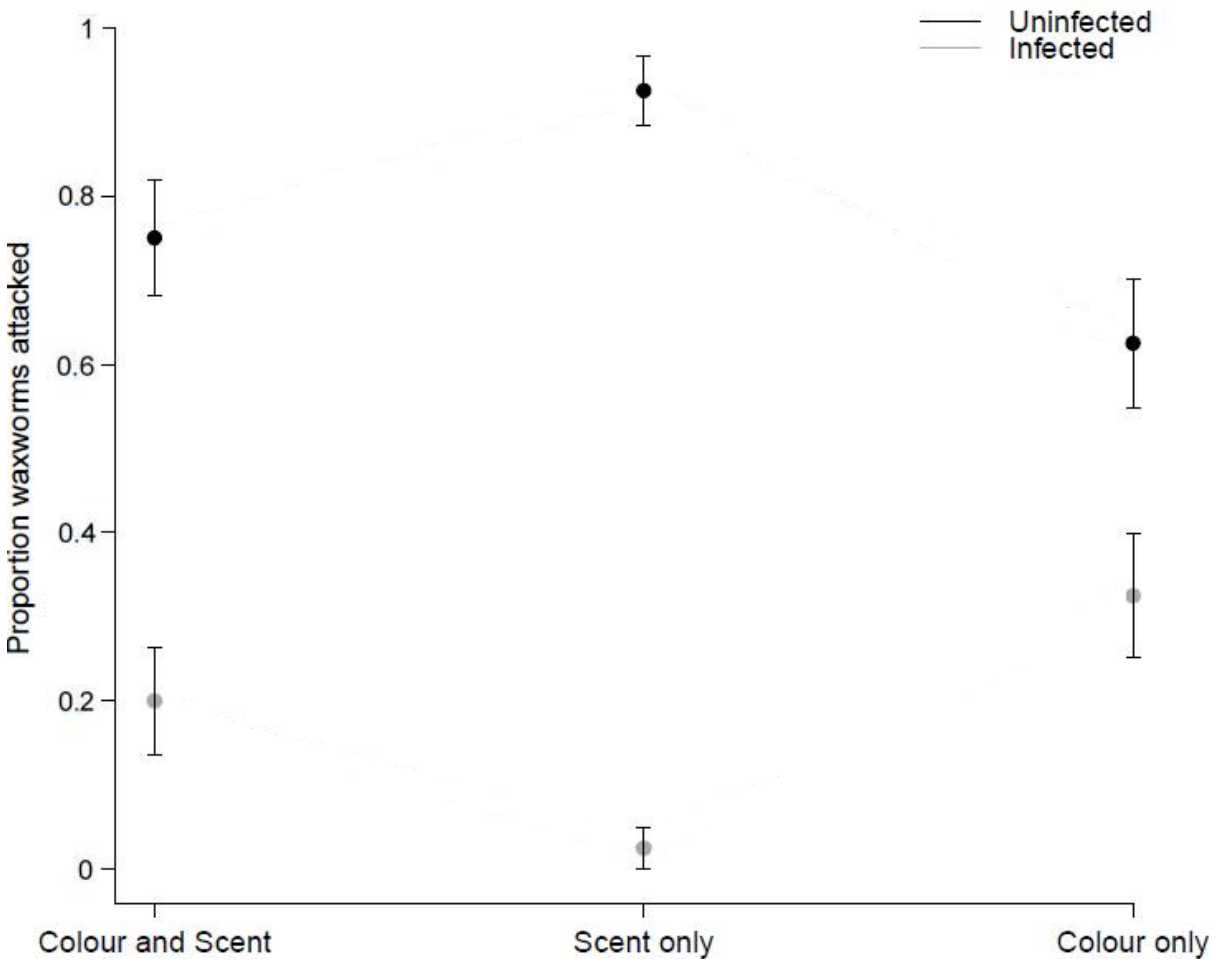

Figure 4.3. Proportion of uninfected or *H. bacteriophora*-infected day 7 post-infection waxworms attacked in the first 4 attacks across the three conditions (Colour and Scent, Scent only and Colour only). Error bars represent 95% confidence intervals.

Consumption rate

Examining the probability of consumption *per* se, there were significantly fewer infected waxworms consumed in the colour and scent condition compared to the scent only condition at days 3 post-infection (Fig. 4.4; z_{80}=-2.622, *P*=0.0087). However, there was no significant difference in the number of infected waxworms consumed at day 5 (Fig. 4; z_{80}=-0.371, *P*=0.710) or day 7 (Fig. 4.4; z_{80}=0.0, P=1) post-infection in either condition. Therefore, having colour and scent is beneficial for infected prey day 3 post-infection, but scent alone provides as good a cue at days 5 and 7 post-infection.

Additionally, examining infected prey that were consumed, following an attack, significantly fewer infected waxworms were consumed in the colour and scent compared to the scent only condition (z_{70}=3.361, *P*<0.001). There was also a significant interaction between the scent only condition and infected prey 5 days post-infection (z_{70}=-2.903, *P*=0.004). However, it is hard to interpret these interactions as on some days there were very few attacks on infected prey.

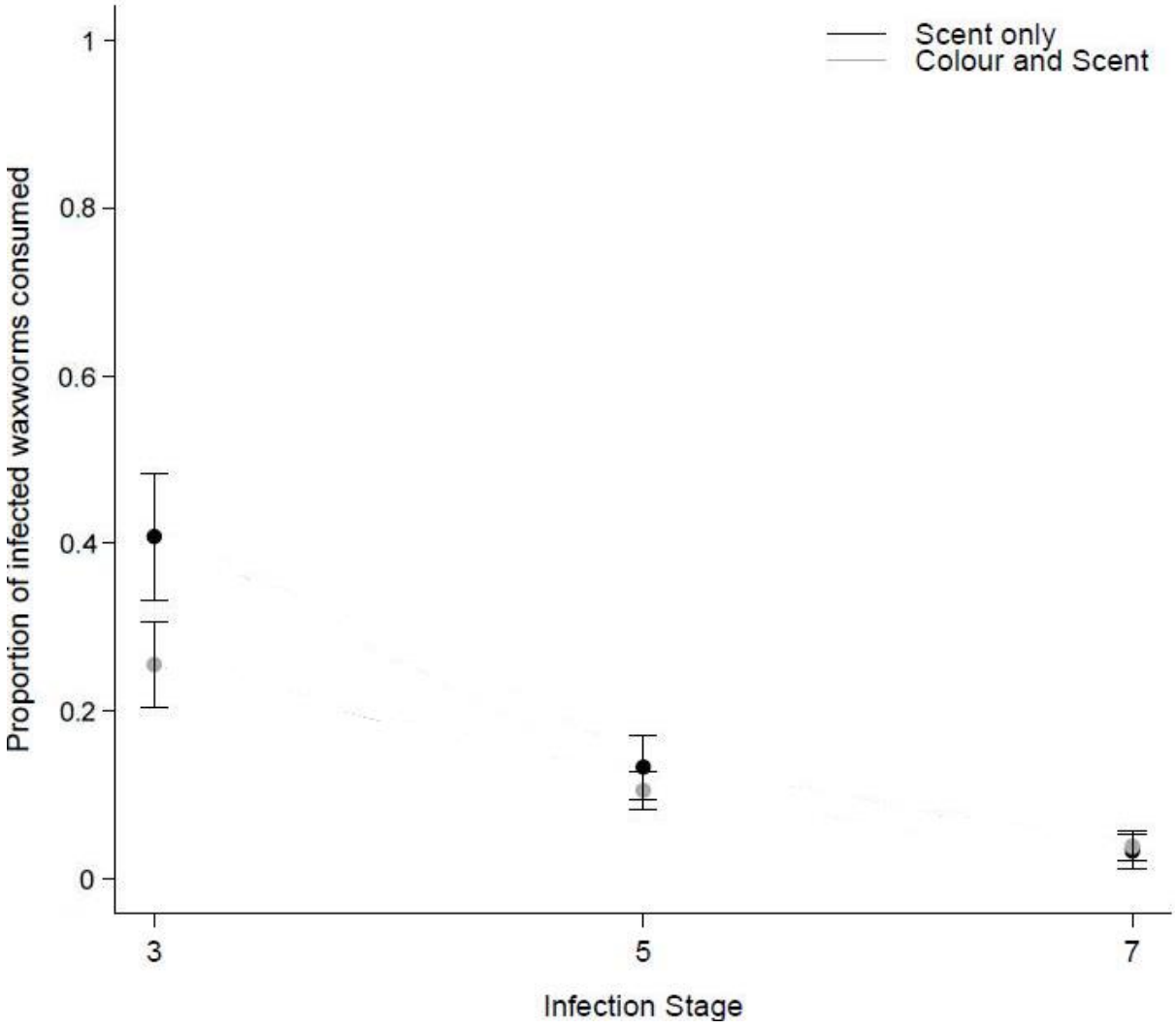

Figure 4.4. Proportion of *H. bacteriophora*-infected waxworms consumed in the colour and scent and scent only conditions across all three infection stages (3, 5 and 7). Error bars represent 95% confidence intervals.

Multimodality

We examined the first prey item attacked for each condition as this was the first initial response of naïve birds to infected or uninfected prey without any reinforcers (i.e. taste) or learning behaviour. We found there was no significant

difference between infected or uninfected prey attacked across infection stage for scent only (Table 4.1; *P*=0.1916), colour only (Table 4.1; P=0.893), scent and colour (Table 4.1; P=0.249) or across all three conditions (Table 4.1; *P*=0.306).

Significantly more uninfected waxworms were attacked first by naïve birds at day 7 post-infection infected waxworms during the scent only condition (χ^2_1=6.4, *P*=0.011), day 5 post-infection approached significance (χ^2_1=3.6, P=0.058) and day 3 post-infection was not significant (χ^2_1=1.6, P=0.206). Therefore, although there was no effect overall of having either a unimodal or multiple defence, there appears to be a benefit to scent for infected day 7 post-infection waxworms.

Table 4.1. Number of uninfected or *H. bacteriophora*-infected waxworms attacked first for each infection stage (3, 5 and 7) for each condition (Colour and scent, Colour only and Scent only).

	Colour and scent			Colour only			Scent only		
	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7	Day 3	Day 5	Day 7
Uninfected	4	8	7	5	6	7	5	8	9
Infected	6	2	3	5	4	3	5	2	1

4.6 Discussion

When examining the effect of multiple defences on predation rates of *H. bacteriophora*-infected waxworms we found mixed effects whereby both signals in concert did not lower attack rates to a greater extent than either signal alone. In terms of attack rate on infected individuals, there was no benefit of multiple signals at

day 3 post-infection but at day 5 post-infection, colour provided less protection than either colour and scent together or scent alone. However, at day 7 post-infection scent alone provided the best protection. Therefore, utilising both colour and smell provides protection at different stages of infection when birds attend to the different signals. However, our study shows similarities to other studies (with larger sample sizes) where colour is the more salient cue over smell (Marples, van Veelen, & Brakefield, 1994) although this could just be an artefact of experimental design as birds were not able to feed in the colour only trial and so had no gustatory feedback. In seven spot ladybirds (*Coccinella semptempunctata*) colour pattern was the most important cue, followed by taste (Marples et al., 1994).

This phenomenon, known as 'over-shadowing', occurs when one component is much more intense than the other and can lead to acquisition speeds of the signal similar to that when both components are present (Ihalainen, Lindstrom, Mappes, & Puolakkainen, 2008; Rowe, 1999). This can also prevent the predator from learning one signal in the presence of another (Siddall & Marples, 2008). Couvillon & Bitterman (1988) found that colour was overshadowed by odour during a 10 minute extinction test following presentation of colour-odour combinations to honeybees. In this study, we found similar effects of scent overshadowing colour in terms of attack at late stages of infection (day 7 post-infection). Colour on the other hand does not seem to provide much protection, unless in combination with scent.

Furthermore, scent and colour only appear to have a strong effect early on during infection in terms of consumption rates of infected individuals with those individuals exhibiting both traits consumed less often than when scent alone is present. However, later on during infection, scent only provides as much protection as colour and scent in concert, suggesting that the scent signal is overshadowing the

colour signal late on in infection. Therefore, although in this case, colour does not seem to have a benefit in its own right, in combination with scent predation and consumption can be minimised and both defences are maintained within this system.

Although both colour and smell are considered in the identification stage of defence, a relatively early stage of the predation sequence, they both confer benefits to the infected individual through reduced attack. By having both defences present at an early stage, although costs of each defence are currently unknown, each defence may be relatively cheap to produce or the nematode-bacterium complex may have more resources available for investment in defence (Speed, 2016, in prep.). However, investment in multiple defences, in this case colour and smell, will be more beneficial when viewed in the context of multiple potential predators (Maleck & Dietrich, 1999; Poitrineau et al., 2003; Rigby & Jokela, 2000; Sih et al., 1998). Individuals are more likely to face multiple rather than single predators and so having multiple barriers in a sequential fashion targeting different predators would vastly improve survival for individuals carrying those defences (Gilman et al., 2012; War et al., 2012). In this system, ground foraging invertebrate and mammalian predators are likely to encounter infected hosts which are likely to prioritise different defences based on their perceptual capabilities, such as invertebrates attending to olfactory signals (Jones et al., 2016) or chemical signals (Gulcu et al., 2012; Zhou et al., 2002) and mice attending to bioluminescent signals (Jones et al., in review).

The two signals we examined are in different sensory modalities and so can be considered in terms of multimodal signalling, whereby components of the signal occur in more than one sensory modality (Rowe, 1999; Scheffer, Uetz, & Stratton, 1996). We examined signals in two different sensory modalities (colour and scent) in this study but infected individuals also bioluminesce (Ffrench-Constant & Bowen,

2000) and also have a chemical defence (Baur et al., 1998; Gulcu et al., 2012; Zhou et al., 2002). Therefore, it is feasible to hypothesise that this nematode-bacterium system is an example of aposematic multimodal signalling. Examining the first prey item attacked we found no benefit for multimodality although there was some protection for day 7 post-infection individuals by the scent only condition whereby more uninfected compared to infected individuals were attacked. However, due to the nature of our experiments, it would be intriguing to test each defence (taste, colour and scent) in a fully factorial design to elucidate if this system is acting in a multimodal signalling manner.

Many studies have examined how odour and/or sound interact with warning colouration to deter predation in domestic chicks (*Gallus gallus domesticus*) utilising artificial combinations of various cues (Marples & Roper, 1996; Rowe & Guilford, 1996, 1999; Siddall & Marples, 2008). The combination of multiple cues often results in a latency to consume novel prey or an increased learning avoidance compared to either cue alone (Marples & Roper, 1996; Siddall & Marples, 2008). For example, Siddall & Marples (2011) found that wild robins (*Erithacus rubecula*) learnt to avoid artificial pyrazine (a common insect warning odour) –treated yellow baits faster compared to those with no odour. However, it is vitally important to understand how these results translate into the natural environment using wild predators (Siddall & Marples, 2011) and natural aposematic signalling prey (Marples et al., 1994). To our knowledge the only study examining multimodal signalling effects of a naturally occurring aposematic insect is that by Marples et al., (1994) whereby the authors tested various combinations of the multimodal signal of the seven-spot ladybird (*Coccinella septempunctata*). Ladybirds were presented to captive Japanese quail (*Coturnix coturnix japonicas*) in treatment combinations with colour pattern, scent

and taste singly, in a two-way combination or the whole insect. Avoidance was maximised when the whole insect was presented, although colour was the most effective single deterrent (Marples et al., 1994).

There are many hypotheses concerning the evolution of multimodal signalling which cover both content and efficacy based hypotheses (see Review, Rowe & Halpin, 2013). Some relate to how multiple signals can increase information value of a signal, the 'multiple messages' or 'back-up' signal hypotheses (Moller & Pomiankowski, 1993). Others relate to how signal components evolve in response to variability within the environment (Candolin, 2003; Hebets & Papaj, 2005) or the perceptual variability in predators relying on signal components in different sensory modalities (Rowe & Halpin, 2013). Multicomponent signalling can also lead to increased detection (Rowe, 1999), improved discrimination (Hebets & Papaj, 2005) and increased learning and memory (Siddall & Marples, 2008). Multimodal signals have also been suggested to act in a sequential manner due to the unique properties of different sensory modalities that make them more detectable at different distances or environmental conditions (Candolin, 2003; Hebets & Papaj, 2005). Some of these hypotheses tie in with literature on multiple defences targeting multiple predators with various barriers acting at different stages of predation.

Overall, this system has the capacity to act in a multimodal fashion through multiple barriers of defence due to the range of defences in different sensory modalities. Various studies have shown adaptive benefits to the range of defences (Baur et al., 1998; Fenton et al., 2011; Gulcu et al., 2012; Jones et al., 2016; Zhou et al., 2002) but few studies have considered these defences in tandem. The defences in this nematode-bacterium occur across multiple stages of predation and we found colour and scent were as beneficial as both signals together at different stages of

waxworm infection in terms of attack and consumption by wild great tits. Therefore, multiple barriers to defence are an effective strategy against predation for this parasitic colony. Furthermore, as multiple predators are likely to encounter nematode-infected individuals, the different defences in this system may act in an aposematic multimodal signalling way to deter predation by predators with different perceptual capabilities at various stages of predation.

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Chapter 5. Parasite-induced bioluminescence deters predation of infected hosts by nocturnal rodent predators

This chapter is currently in review at Behavioural Ecology.

5.1 Author contributions

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David Clarke provided some thoughts for the discussion. Mike Speed and Andy Fenton provided comments on the manuscript and discussion of ideas. Jane Hurst provided comments on the manuscript, assisted with experimental design and discussion of ideas. I helped design the study, conducted the data collection and analysis and wrote the manuscript.

5. 2 Abstract

Anti-predator defences are ubiquitous in nature with aposematism a common and well-studied example. Aposematism normally combines a repellent defence, such as

a toxin with a warning signal, usually visual, olfactory or acoustic. There is now increasing evidence that bioluminescence can act as an aposematic (warning) signal to deter predation of prey that have chemical defences. We examine a potentially novel example of such signalling; the bioluminescence of infected insect cadavers induced by infection with the parasitic nematode *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*. This nematode-bacterium complex infects soil-dwelling hosts within which it reproduces for around two weeks before new infective nematodes emerge. During this incubation period the insect cadaver, and therefore the nematode-bacterium complex, is susceptible to predation, which is fatal for the developing parasites. We hypothesise that bioluminescence in this system acts as a warning signal to deter predation of infected hosts by nocturnally active, foraging predators. We tested both an olfactory and bioluminescent deterrent within this system by assessing the behavioural response of house mice (*Mus musculus*) towards insect prey that were either infected or uninfected under different light conditions. We found that mice did not respond to an olfactory cue but did spend less time near bioluminescent prey, indicating an avoidance of prey based on a luminescent signal, rather than an olfactory cue. Bacterial symbionts in this system may have evolved exaggerated luminescent signals in order to protect a parasitic colony from predation.

5.3 Introduction

Predation is an important process in the natural world with few animals immune to the risk of predation at some point of their life cycle. Anti-predator

defences are therefore a widespread and commonly studied occurrence (Ruxton, Sherratt, & Speed, 2004). One particular example of anti-predatory defences is aposematism, the association of a warning stimulus such as a colour, sound or odour with a repellent defence, such as a toxin (Poulton, 1890). There are very many examples of aposematically signalling animals in nature, including insects and mammals (Caro, 2005; Cott, 1940; Edmunds, 1974; Ruxton et al., 2004). Many studies have examined aposematism as a combination of toxin with either colour (Guilford, 1990b; Roper, 1990), sound (Hristov & Conner, 2005) or smell (Eisner & Grant, 1981; Jetz, Rowe, & Guilford, 2001; Rowe & Guilford, 1996, 1999; Siddall & Marples, 2011) or more than one in concert (Marples & Roper, 1996; Marples, Van Veelen, & Brakefield, 1994; Siddall & Marples, 2008). However a lesser studied and intriguing form of aposematism occurs with a bioluminescent warning signal. Bioluminescence is the ability to produce light through biochemical reactions between luciferases and luciferins or photoproteins (Hastings & Wilson, 1998), and is rare in terrestrial environments (Haddock, Moline, & Case, 2010). However, there is evidence that bioluminescence could act as an aposematic signal to deter predation (De Cock & Matthysen, 1999; Marek, Papaj, Yeager, Molina, & Moore, 2011; Matthysen & De Cock, 2001; Underwood, Tallamy, & Pesek, 1997). Furthermore, bioluminescence may conceivably be a deterrent in itself, its novelty in terrestrial environments causing enhanced wariness in foraging animals (Marples & Mappes, 2010).

A well-known example of terrestrial bioluminescence is that of the glow-worm (Lloyd, 1971). Although the primary role of bioluminescence in the glow-worm system is in mate signalling and selection, it may have a secondary role as an aposematic warning signal of its unpalatability. Glow worm larvae (*Lampyris*

noctiluca, Lampyridae) are unpalatable to birds (Matthysen & De Cock, 2001) and are avoided by house mice (*Mus musculus*) (Underwood, Tallamy, & Pesek, 1997) and toads (*Bufo bufo*) (De Cock & Matthysen, 1999) compared to non-glowing prey. Additionally, bioluminescence in luminescent millipedes has been demonstrated to deter rodent predators from predation (Marek et al., 2011). Glow-worms biosynthesise the enzymes needed for bioluminescence themselves. It is much rarer for bioluminescence to be produced by bacterial symbionts in the terrestrial environment (Haddock et al., 2010). A rare example of this occurs between a specific genus of nematode such as *Heterorhabditis bacteriophora* and its luminous symbiotic bacterium *Photorhabdus luminescens* (Nealson & Hastings, 1979). These soil-dwelling obligate insect parasites infect soft-bodied insect larvae (Kaya & Gaugler, 1993), ejecting the symbiotic bacteria which kills the insect before both nematodes and bacteria reproduce within the host (Poinar, 1975). Between infection and release of new infectious juveniles into the environment, however, there is a lag phase of up to 20 days when infected insects are vulnerable to predation (Clarke, 2008), which would be fatal to the nematode (Fenton, Magoolagan, Kennedy, & Spencer, 2011). Therefore, protection during this vulnerable stage is important for successful nematode reproduction and propagation. As Jones et al (2015) recently argued, since the parasites disable the host's anti-predator defences when they kill it, they need to replace them with alternative protection from predators and scavengers.

Inkeeping with aposematism, nematode-infected insects use a combination of chemical defence (Baur, Kaya, & Strong, 1998; Gulcu, Hazir, & Kaya, 2012; Jones, Fenton, & Speed, 2015; Zhou, Kaya, & and Goodrich-Blair, 2002), colour change (hosts turn dark red) (Fenton et al., 2011) and foul-smelling odour (Jones, Fenton, &

Speed, 2016) to deter predators. Jones et al. (2015) were the first to show that the foul-smelling odour of infected individuals alone is enough to deter predation of infected waxworms by invertebrate predators (beetles) from early to late nematode infection. As the olfactory signal was such a strong deterrent with invertebrate predators, it has the potential to act as a deterrent within its own right, in organisms with more advanced olfactory systems. Therefore, further study into this poorly understood olfactory signal will complement previous studies elucidating the role of the olfactory cue within this system.

One other notable feature of insect cadavers infected with this nematode-bacterium complex is that they bioluminesce for the first three days post-infection (Daborn, Waterfield, Blight, & Ffrench-Constant, 2001). However, the role(s) of this bioluminescence is not yet understood. There is accumulating evidence that this bioluminescence plays an important functional role during the symbiotic association with the nematode (Joyce, Lango, & Clarke, 2011; Lango & Clarke, 2010; Skjerning, Roghanian, Gerdes, & Clarke, 2016). For example, bioluminescence (an O_2 and NADH-consuming biochemical reaction) may be important for maintaining appropriate redox conditions for the developing nematodes within the insect cadaver (Clarke, 2014). However, as in the glow-worm system, it may also play a secondary role as a predator deterrent, either as an aposematic signal, or a deterrent in its own right. Whilst the other defences mentioned above accrue over time, bioluminescence occurs rapidly, but transiently, following death of infected insects and at a time that other defences such as olfactory deterrence and toxins are not present (Fenton et al., 2011; Jones et al., 2016). Hence, we hypothesise that bioluminescence could act as an early defence system during this time, while the other defences are building up. Furthermore, bioluminescence could also be acting to deter nocturnal predators

specifically, which would not necessarily be able to perceive other visual signals whilst foraging.

We recently reported that olfactory cues had deterrent effects on foraging beetles so we investigated whether olfaction and/or bioluminescent cues could act to deter mammalian predators. We tested this hypothesis using experimental choice trials involving a nocturnal, ground foraging predator, the house mouse (*M. musculus domesticus*). As mouse visual systems are dichromatic and relatively poor (Jacobs, Williams, Cahill, & Nathans, 2007) we tested for any deterrence arising from both the olfactory and bioluminescent cues from *H. bacteriophora* infected and uninfected waxworms under two different light conditions. We used a red light to assess the role of olfactory cues in influencing prey choice, in the absence of a bioluminescent cue, for 3 stages of infection, days 3, 5 and 7 post-infection. We then used a UV light to assess the role of a bioluminescent signal in influencing prey choice in a single infection stage, day 3 post-infection, when bioluminescence is present. During both trials prey were presented in a non-contact two-choice experiment and we examined various exploratory behaviours towards the two prey items. Overall we show that mice preferred uninfected over infected prey, and that this choice was driven by the bioluminescent cue of infected prey, rather than an olfactory cue.

5.4 Methods

We ran two experimental trials, the first to test for evidence of an olfactory deterrent from infection under red light conditions and the second to test for a

bioluminescent deterrent under UV light conditions. Rodent housing, visual preference tests and data analysis were consistent across trials and experiments.

Rodent housing

Female wild-stock house mice (*Mus musculus domesticus*) were housed in 45 x 28 x 13cm cages (MB1, North Kent Plastics, UK) in single-sex family groups (2-5 sisters per cage during the test period). Males were housed singly in 43 x 11.5 x 12 cm cages (M3, North Kent Plastics, UK). Subjects were naïve predators and were only utilised for a single trial.

Throughout, all animals were housed on a reversed 12:12h light cycle with lights off at 0800. Mice were maintained on Corn Cob Absorb 10/14 substrate with paper wool nest material and *ad libitum* access to water and food (Lab Diet 5002 Certified Rodent Diet, Purina Mills, St Louis, MO, USA). Cardboard tunnels and plastic mouse houses were provided for home cage enrichment. All animal care protocols were in accordance with the University of Liverpool Animal Welfare Committee requirements and UK Home Office guidelines for animal care.

Preference tests

Tests were conducted in clean 45 x 28 x 13cm arenas (MB1 cage base fitted with a perforated Perspex lid) with two Perspex tubes (internal diameter = 27 mm, length = 19.7 cm) spaced 16.5 cm apart (Fig. 5.1). Both tubes were fitted with a mesh cap (45 mm x 38 mm, internal diameter = 32 mm) covered in black electrical tape to obscure any visual cues of the prey to the human observer during video playback and analysis. Mice were able to sniff and see prey located at the entrance to each tube but were not allowed to contact or taste it. An additional external cue

was provided by a radio in the same location within the room to produce a low, even background noise to ensure mice were not disturbed by extraneous noise during the trials.

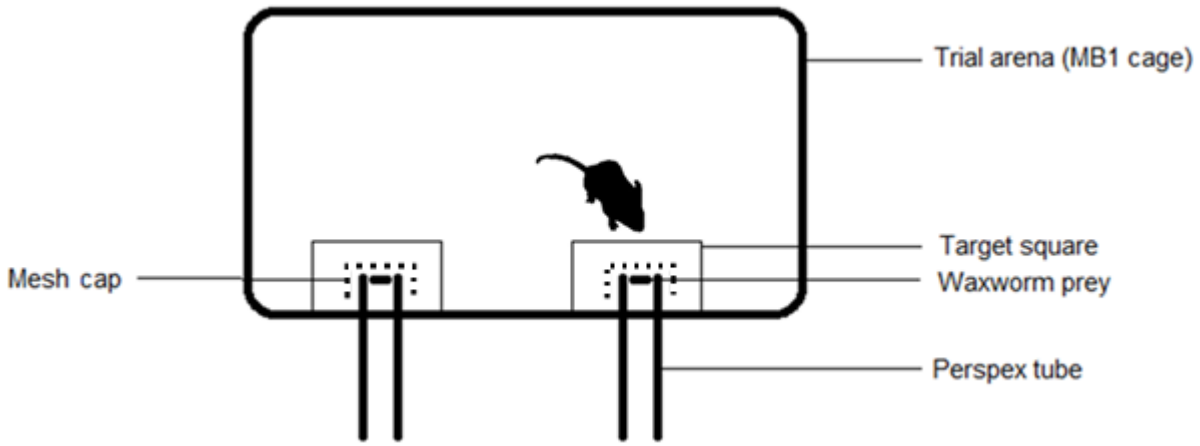

Figure 5.1. Aerial view of the test arena (MB1 cage) with two Perspex tubes situated 16.5 cm apart, each containing a waxworm located behind a mesh cap. Black electrical tape obscured the presented items from the person observing. Thin black lines represent the area in which animals were scored as near to the prey.

Each test consisted of two stages, an initial 5 minute habituation to the test arena, followed by a 10 minute test phase. During habituation, the tubes contained no prey but during the test phase, each tube received either a *H. bacteriophora* infected waxworm or an uninfected waxworm which were randomised across the tubes.

We utilised a CCTV security camera that was sensitive to UV and red light to film the trials. Subject behaviour towards the two tubes was recorded remotely on DVD for both stages and transcription of DVD recordings was carried out blind to the position of the test stimulus during each trial using an event recording program. Our focus was on any interest in, or avoidance of, infected or uninfected waxworms and

so the behaviours we measured were time investigating the mesh in front of each waxworm (sniffing or gnawing at the mesh) and time near the mesh but without active investigation (body within a 9 cm x 7.5 cm area around each mesh cap).

Data analysis

As the data were not normally distributed and could not be transformed appropriately, non-parametric Wilcoxon matched-pair tests were utilised in R. In accordance with ethical requirements to minimise the number of test animals utilised, each test used $N = 12$ subjects of each sex, which was sufficient to show an avoidance behaviour in the bioluminescence test.

Quantifying Bioluminescence

We ran two experiments to test for an olfactory deterrence under red light conditions and bioluminescence deterrence under darkness, henceforth UV light conditions. A UV light was utilised to ensure mice were visible during subsequent analysis of video records although the experiment was in darkness. We quantified the level of bioluminescence in darkness (UV light conditions) and under red light for each experiment. We utilised day 3 post-infection prey for quantification as this is the period of time when bioluminescence occurs.

Nematode infection

Waxworm larvae (3rd instar *Galleria mellonella*) were infected with the nematode strain *Heterorhabditis bacteriophora* TT01 (provided by D. Clarke and S. Joyce, UCC, Ireland) using standard techniques in which 10 waxworms were placed on filter paper with 1000 IJs/mL of nematode culture in a 90-mm petri dish (Kaya & Stock, 1997). Waxworms were analysed fresh at day 3 post-infection. Uninfected control waxworms were frozen for 30 minutes in a -80˚C freezer prior to analysis to kill the waxworms only.

Quantification

An IVIS® Spectrum In Vivo Imaging System (Perkin Elmer, Massachusetts) was utilised for all measurements. Bioluminescence was measured for 4 dishes simultaneously with an exposure of 0.5 seconds, a binning factor of 8 and a field of view of 22.8 cm with no excitation filters for those under complete darkness. The same measurements were utilised for those under red light conditions but an emission filter of 740 nm was used to replicate red light conditions during the trial (average peak wavelength = 735 nm, Colourglaze lightbulb, Crompton). Regions of Interest (ROIs) were constructed in Living Image software (Version 4.5.2.18424, Perkin Elmer) which encompassed a single petri dish. Total flux for each ROI was then divided by the total number of bioluminescent or non-bioluminescent waxworms in each dish to give an average total flux value for each petri dish, which was then averaged across the total number of petri dishes in a treatment.

Experiment 1: Olfaction

We ran separate olfactory trials for infected waxworms at days 3, 5 and 7 post-infection against uninfected controls that were either freshly killed or were killed and had decayed for the same length of time as infected waxworms. Both types of control were used to ensure that any avoidance of infected waxworms was not caused by the smell of decay. This experiment was run under red light conditions, to ensure that bioluminescence was not visible for day 3 post-infection waxworms.

Subjects

Experimental subjects were 12 captive-bred adult female house mice and 12 captive-bred adult males aged 4-26 months, in good health and naïve to waxworms.

Nematode infection

Infected waxworms were set up as before (See 'Quantifying Bioluminescence). Infected waxworms were utilised at days 3, 5 and 7 post-infection. Control freshly killed uninfected waxworms were freeze-killed for 30 minutes in a -80˚C freezer on the day of the trial. Infected waxworms die two days following nematode infection, so control uninfected but decayed waxworms (hereafter, decayed waxworms) were freeze-killed for 30 minutes in a -80˚C freezer at days 1, 3 and 5 to match the timing of death and decay of infected prey at days 3, 5 and 7 respectively.

Experiment 2: Bioluminescence

The bioluminescent trial was run under UV light to ensure the bioluminescent signal was visible and freshly killed uninfected waxworms were utilised as controls.

Subjects

Experimental subjects were 12 captive-bred adult female *Mus musculus domesticus* and 12 captive-bred adult males aged 11 months and naïve to waxworms.

Nematode infection

Infected waxworms were set up as before (See *'Quantifying bioluminescence'*) and utilised at day 3 post-infection. Additionally, on the day of the tests fresh uninfected waxworms were freeze-killed for 30 minutes in a -80˚C freezer and utilised as the non-bioluminescent visual prey.

5.5 Results

Firstly, we examine the quantification of bioluminescence in response to the two light conditions. We then discuss the results in relation to the two experiments, firstly olfaction, split by infection stage, and then bioluminescence. Across all trials and experiments there was no pre-existing bias in either time near the tunnel or time investigating the tunnel within each test (Wilcoxon matched pair test, $p > 0.05$).

Quantifying Bioluminescence

Bioluminescence of day 3 post-infection waxworms was not visible under red light conditions, but was under UV light conditions (complete darkness) (Fig. 5.2). Under UV light, the strength of bioluminescence of waxworms infected with *H. bacteriophora* TT01 at 3 days post-infection was over 2 orders of magnitude greater than that of uninfected controls (Fig. 5.2). The mean bioluminescent total flux for each infected waxworm was 1.2 x 10^{10} photons per second, compared to just 4.5 x 10^{7} photons per second (i.e not-bioluminsecing) for uninfected waxworms. Under red light conditions, the mean total flux for each infected waxworm was 4.0 x 10^{7} photons per second which is much lower than that for bioluminescent waxworms under UV conditions but comparable with uninfected controls. We can therefore be confident that bioluminescence was not visible during the first experiment testing for an olfactory cue but was visible for the second experiment testing for a bioluminescent signal.

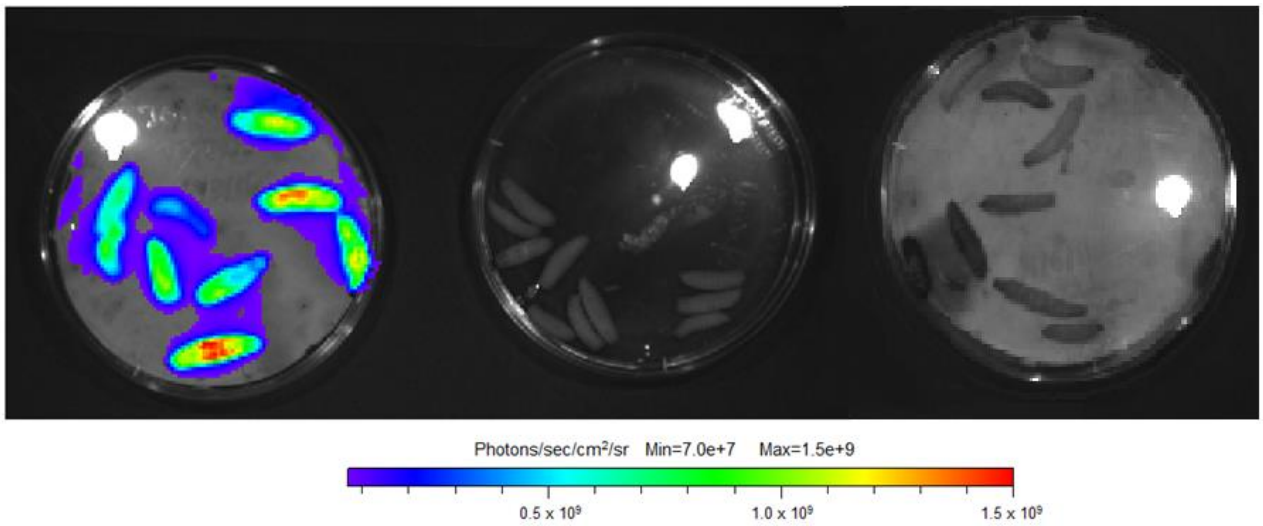

Figure 5.2. *H. bacteriophora* infected waxworms under UV (left) and red light (right) conditions against an uninfected control (centre). Waxworms were imaged using an IVIS Spectrum In Vivo Imaging System (Xenogen) with no emission filter (for UV light conditions) and an emission filter of 740nm (for red light conditions). For both images shown, the colour scale ranges from blue (just greater than the background noise; set to 7.0×10^7 photons/s/cm^2/sr) to red (at least 1.5×10^9 photons/s/cm^2/sr).

Experiment 1: Olfaction

a) Day 3 post-infection

There was no significant difference in the time mice spent investigating the mesh in front of the infected waxworm or uninfected control (Fig. 5.3; sniffing and gnawing behaviours towards decayed waxworm, V = 145, N=24, $p = 0.900$; fresh waxworm, V = 192, $p = 0.241$), and no difference in the time spent near these prey items when not actively investigating (Fig. 5.3; decayed waxworm, V = 196, N=24, $p = 0.197$; fresh waxworm, V = 202, N=24, $p = 0.143$). Therefore, any olfactory cue of *H. bacteriophora*-infected waxworms did not significantly instigate any aversion or discriminatory behaviour.

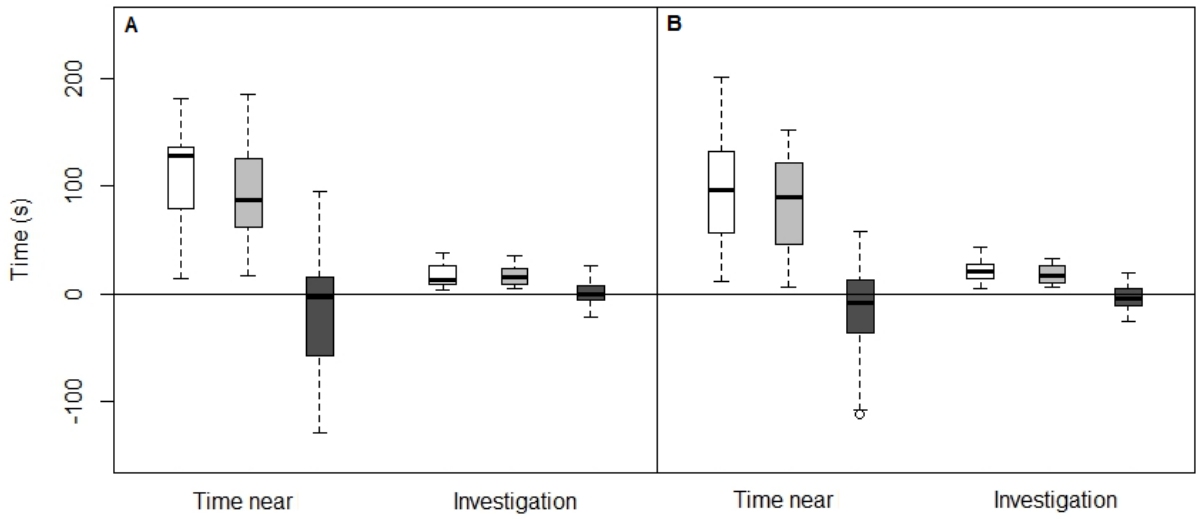

Figure 5.3. Response to prey 3 days post-infection under red light. Mice were presented with a waxworm infected 3 days prior to the trial versus an uninfected control which was either A) killed and left to decay for 1 day to match the death and decay of the infected prey or B) freshly killed. Boxplots show median and interquartile range with 1.5 x IQR whiskers for the infected (white), uninfected (light grey) and difference in response (uninfected-infected: dark grey).

Day 5 post-infection

There was no significant difference in the time mice spent investigating the mesh in front of the infected waxworm or uninfected control (Fig. 5.4; sniffing and gnawing behaviours towards decayed waxworm, V = 125, N=24, p = 0.491; fresh waxworm, V = 143, N=24, p = 0.855), and no difference in the time spent near these prey items when not actively investigating (Fig. 5.4; decayed waxworm, V = 179, N=24, p = 0.422; fresh waxworm, V = 153, N=24, p = 0.943). Therefore, any olfactory cue

of *H. bacteriophora*-infected waxworms did not instigate any aversion or discriminatory behaviour.

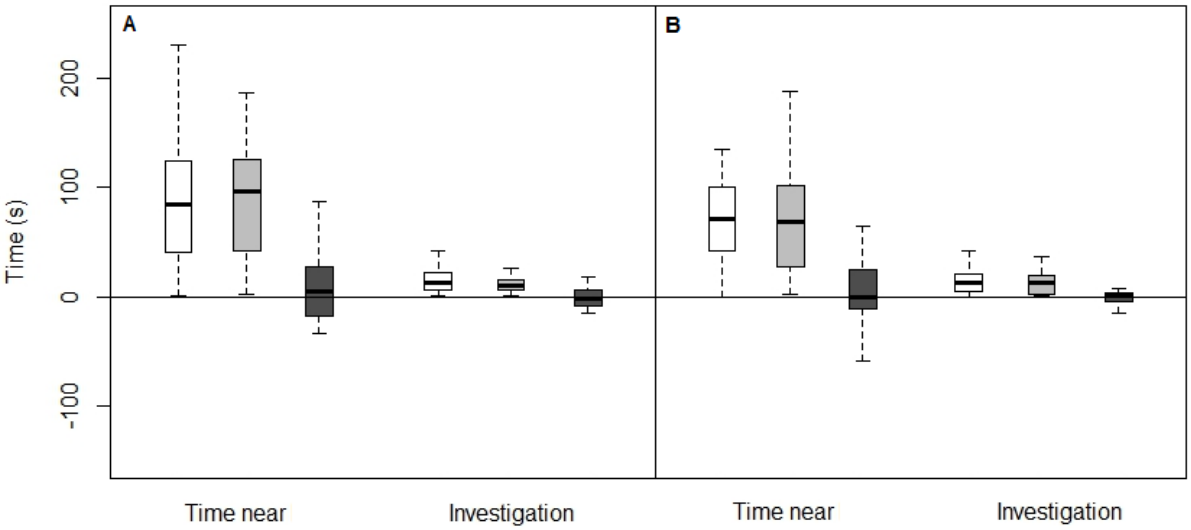

Figure 5.4. Response to prey 5 days post-infection under red light. Mice were presented with a waxworm infected 5 days prior to the trial versus an uninfected control which was either A) killed and left to decay for 3 days to match the death and decay of the infected prey or B) freshly killed. Boxplots show median and interquartile range with 1.5 x IQR whiskers for the infected (white), uninfected (light grey) and difference in response (uninfected-infected: dark grey).

b) Day 7 post-infection

There was no significant difference in time spent near infected versus uninfected prey whether using an uninfected control that was decayed or fresh (Fig. 5.5; decayed waxworms, V = 142, N=24, p = 0.833; fresh waxworm, V = 125, N=24, p = 0.491). However, investigation time differed significantly, with less investigation of the infected than the uninfected prey regardless of decay (Fig. 5.5; decayed

waxworm, V = 72, N=24, $p = 0.025$; fresh waxworm, V = 63, N=24, $p = 0.012$). Therefore, although the olfactory cue 7 days post-infection did not instigate any aversion, it did instigate some discriminatory behaviour as mice spent less time investigating an infected over an uninfected waxworm in both trials (sniffing or gnawing at the mesh in front of the prey).

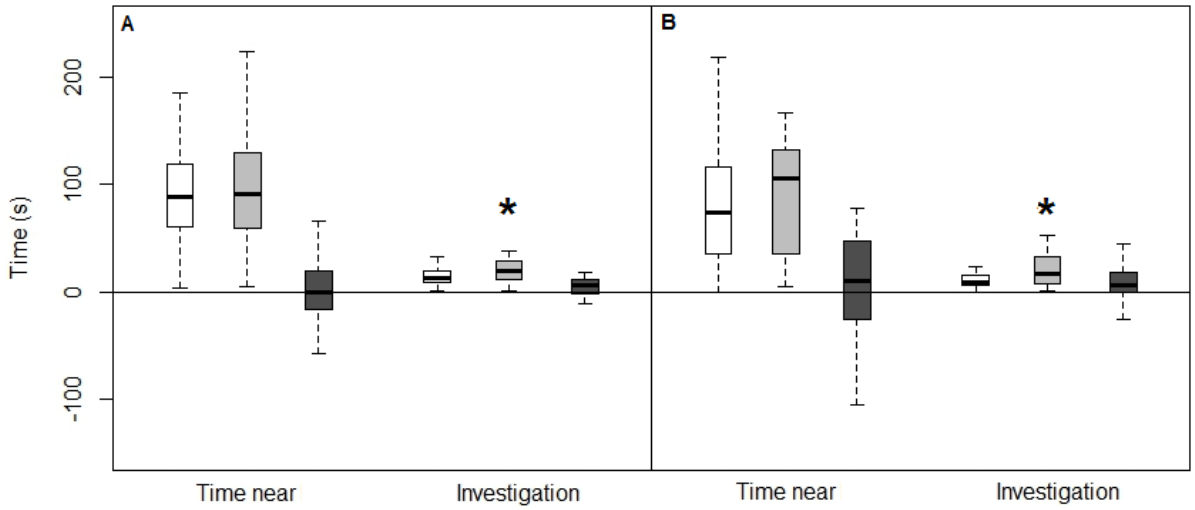

Figure 5.5. Response to prey 7 days post-infection under red light. Mice were presented with a waxworm infected 7 days prior to the trial versus an uninfected control which was either A) killed and left to decay for 5 days to match the death and decay of the infected prey or B) freshly killed. Boxplots show median and interquartile range with 1.5 x IQR whiskers for the infected (white), uninfected (light grey) and difference in response (uninfected-infected: dark grey). * $p < 0.05$.

In conclusion, there were no avoidance behaviours shown towards infected prey over uninfected prey during the olfactory trials, although there was evidence of some discriminatory behaviour at the later stage of infection (day 7 post-infection) whereby mice sniffed and gnawed less at the mesh in front of infected compared to

uninfected waxworms. These results were repeatable when carried out using fresh or decayed waxworms as uninfected controls, suggesting that the scent of a dead infected waxworm alone does not cause aversion.

Experiment 2: Bioluminescence

House mice spent significantly more time near the non-bioluminescent prey compared to the bioluminescent prey (Fig. 5.6; $V = 56$, $p = 0.006$). Although investigation of the non-bioluminescent prey also tended to be higher, this did not reach significance (Fig. 5.6; $V = 91$, $p = 0.095$). As olfactory cues did not produce any direct aversion (Fig. 5.3.) and bioluminescence was not visible under red light conditions (Fig. 5.2), preference to spend more time near uninfected prey must be due to the bioluminescent signal.

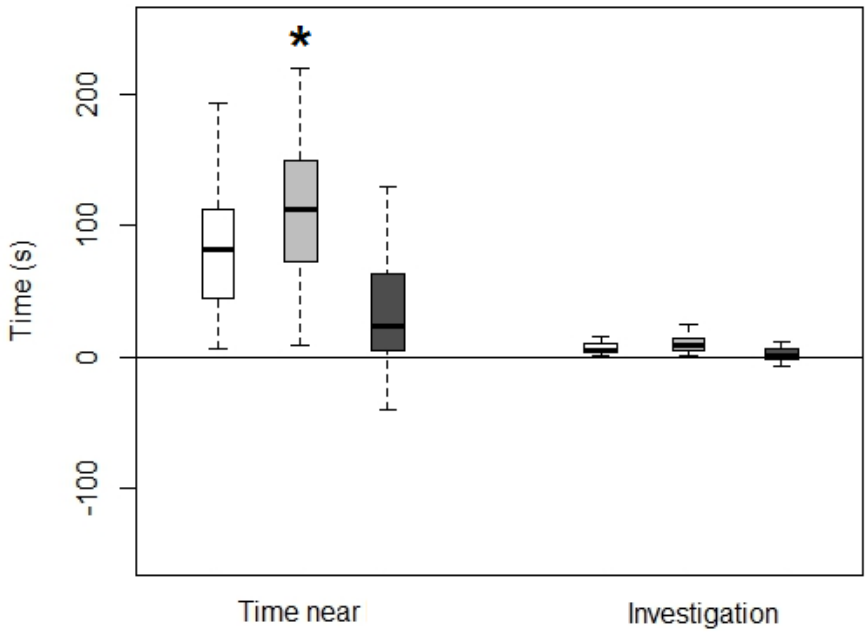

Figure 5.6. Response to prey 3 days post-infection under UV light conditions. Mice were presented with a waxworm infected 3 days prior to the trial versus a freshly

killed uninfected control. Trials were run under UV light to ensure the bioluminescent signal was visible. Boxplots show median and interquartile range with 1.5 x IQR whiskers for the infected (white), uninfected (light grey) and difference in response (uninfected-infected: dark grey). * $p < 0.05$.

5.6 Discussion

Unlike other studies examining olfaction in this system (Jones et al., 2016), we found the foul-smelling odour produced by nematode infected insects did not appear to be aversive to mice, and thus may not act as a deterrent to these rodents. However there was evidence of discriminatory behaviours towards later stage infections (day 7 post-infection), whereby mice spent less time sniffing and gnawing at the barrier to reach infected insects. It therefore seems that house mice may not be particularly sensitive to the odour cue, as observed in beetles, and may utilise another cue for avoidance of nematode-infected insects.

Our data provide evidence that bioluminescence within this system may act on its own to deter predation on *H. bacteriophora*-infected hosts by rodent predators. Although there was no significant difference in investigatory behaviours (sniffing or gnawing), house mice spent significantly more time by the tube with non-bioluminescent, uninfected prey items, compared to the tube with bioluminescent, infected prey items. Notably no preference was observed in the absence of the bioluminescent signal under red light conditions (i.e., in the presence of olfactory cues alone when bioluminescence was not visible). Infected prey are known to have a chemical defence and feeding deterrent (Baur, Kaya, & Strong, 1998; Gulcu, Hazir, & Kaya, 2012; Jones et al., 2016). However, this takes effect later on during infection (Baur et al., 1998; Jones et al., 2016), with early infections quite vulnerable to

predation. Unlike traditional views of aposematism, whereby a colour cue (normally some sort of pigment) is backed up by a chemical defence, in this case the bioluminescent signal occurs before toxins build up. It is therefore plausible to suggest that bioluminescence acts as a deterrent in its own right, causing mice to avoid the infected prey without requiring a chemical defence.

Our results also support previous studies whereby both mouse and toad predators use light cues to avoid distasteful prey (De Cock & Matthysen, 1999; Matthysen & De Cock, 2001; Underwood et al., 1997). However, unlike those previous studies, bioluminescence in this system is produced by a bacterial symbiont (Haddock et al., 2010). To our knowledge this is the first case of parasite-induced bioluminescence deterring predation of infected hosts in a terrestrial environment. Bioluminescence might be utilised in this system for a number of protective functions. First, as the hosts that the nematodes infect are soil-dwelling, a large number of predators are likely to encounter them whilst foraging. Many predators have been shown to avoid infected hosts based on either the visual cue (Fenton et al., 2011), chemical defence (Baur et al., 1998; Gulcu et al., 2012; Jones et al., 2016) or odour cue (Jones et al., 2016), but these studies have mostly examined diurnal predators. Many predators forage at night when these cues may be rendered useless and so bioluminescence could be a viable means to deter nocturnal predation. Mice in our study were able to use this light cue to avoid infected insects preferably over the odour cue, which only elicited some discriminatory behaviour towards later-stage infections. Additionally, infections are vulnerable early on and it takes a few days for the toxins and colour change to build up to sufficient strength to deter predation (Clarke, 2008; Fenton et al., 2011; Jones et al., 2016). Having an

additional defence early on during infection will act as another barrier to predation, reducing the risk of host predation and ultimately nematode-bacterium death.

Although we propose that bioluminescence in this system could act as a deterrent signal, there is also evidence that bioluminescence plays a biochemical role in the nematode-bacterium association. Light production in *Photorhabdus* is mediated by a single genetic locus, the *luxCDABE* operon. This operon encodes all of the enzymes required to carry out a well-characterised biochemical pathway that uses a fatty acid, O_2 and NADH as substrates to produce a photon of light. Indeed the luciferase enzyme (encoded by the *luxA* and *luxB* genes) has a very high affinity for O_2 and therefore light production may result in a limitation in the availability of O_2 within the insect cadaver (Clarke, 2014). This bioluminescence-dependent niche modulation may be important during the bacteria-nematode association, although this has not yet been tested. Nevertheless, even if bioluminescence primarily plays a role in maintaining the internal environment within an infected host, we have also been able to demonstrate an additional adaptive value to bioluminescence; that of predator avoidance. As seen with glow-worms (De Cock & Matthysen, 1999, 2003), although bioluminescence has evolved for a different primary purpose, it may still act as a deterrent in its own right.

An interesting question is whether this bioluminescence is enhanced by the bacteria during this early stage of infection to deter predation or is simply the baseline luminosity produced as a result of the maintenance required for the internal conditions. Additionally, what is it about glowing *per* se that causes avoidance by predators and protects the nematode within this system? It could be the sheer novelty of a glowing food item that deters predation, or neophobic reactions to prey that are glowing. There are also a number of strains of this nematode which have

varying degrees of bioluminescence (David Clarke, personal communication) and we could hypothesise that those with increased bioluminescence may persist in sympatric environments with large numbers of nocturnal predators. Although not studied here, different strains of this nematode with varying levels of bioluminescence could be tested to determine whether the strength of avoidance correlates with the strength of bioluminescence. Furthermore, a phylogenetic analysis of the distribution of both more bioluminescent strains and nocturnally active predators could provide insight into the diversity and application of bioluminescence seen within this nematode species.

Overall, our work adds further support to the role of bioluminescence as a deterrent and avoidance signal. However, to our knowledge, this is the first demonstration that a novel form of bioluminescence induced by a bacterial symbiont is used to deter predation of parasite infected hosts, to ensure propagation of this nematode-bacterium complex. As such we suggest that bioluminescence within this system can act as an additional barrier to predation during early infection, particularly against nocturnally foraging predators, to protect the parasitic community inside infected hosts.

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Chapter 6. Conclusions and future work

6.1 Conclusions

This thesis elucidates the roles of anti-predator defences employed by the nematode-bacterium system *Heterorhabditis bacteriophora-Photorhabdus luminescens*. These novel forms of host manipulation provide critical defence from a wide range of potential predators. Conclusions specific to each chapter are highlighted within chapter discussions so this chapter will therefore provide an integrative discussion of the results more broadly.

Parasite manipulation to increase transmission is a common strategy utilised by parasites to reach their definitive host where they reproduce (Dobson, 1988). There are a number of ways in which this is achieved and perhaps the most common relates to trophic transmission, where parasites manipulate the behaviour or appearance of their intermediate host to increase its susceptibility to predation by a definitive host (Lafferty, 1999). Although an effective strategy, a much rarer and less studied phenomenon occurs whereby parasite manipulation is utilised to avoid predation. This scenario occurs in the nematode-bacterium complex *Heterorhabditis bacteriophora-Photorhabdus luminescens*. This thesis expands on a small number of existing studies into this (or a related) system examining the ecological aspects of different defences induced by the infecting nematode-bacterium complex, either chemical (Baur, Kaya, & Strong, 1998; Gulcu, Hazir, & Kaya, 2012; Zhou, Kaya, & Goodrich-Blair, 2002) or visual (Fenton, Magoolagan, Kennedy, & Spencer, 2011), by examining the roles of the multiple defences in this system and how they perform in concert in a multimodal fashion.

Firstly, I tested the role of colouration changes to determine whether infected individuals were signalling aposematically or cryptically. Aposematism is an effective strategy to reduce predation by using a warning signal, normally colour, sound or smell (Cott, 1940; Poulton, 1890; Wallace, 1867). However, infected individuals are often found in the soil substrate and would appear cryptic against their background (Chapter 2). I found that infected individuals at all stages of infection suffered fewer attacks from avian predators (also reflected in Chapter 4) and were consumed less when conspicuous against their background, in keeping with the literature on aposematism whereby predators avoid unpalatable prey and do so more quickly when aposematic rather than cryptic (Guilford, 1986; Speed, 2000). However, crypsis, in combination with other defences, may play a role in this system for other potential predators of infected hosts which lack extensive visual systems such as arthropods.

Infected individuals produce a strong-smelling odour during laboratory infections, and I tested whether this acts as an anti-predator deterrent, alongside the chemical defence with ground beetles (Chapter 3). Olfactory aposematism has received increasing attention with pyrazine a textbook example of a naturally occurring common insect warning odour deterring predation of warningly coloured prey items (Eisner & Grant, 1981; Jetz, Rowe, & Guilford, 2001; Marples & Roper, 1996; Rowe & Guilford, 1996; Siddall & Marples, 2008, 2011). Previous work has shown the presence of a chemical defence in infected individuals (Baur et al., 1998; Gulcu et al., 2012; Zhou et al., 2002) and I found that it reduced feeding rates of ground beetles on infected hosts except in the early stages of infection, suggesting some vulnerability to early stage infections shortly following death. This supports previous work highlighting a higher level of vulnerability in early stage infections

(Baur et al., 1998; Fenton et al., 2011; Gulcu et al., 2012). I then tested whether the strong-smelling odour of infected hosts benefited the parasitic colony in terms of predator avoidance, as seen with pyrazine during laboratory trials (Eisner & Grant, 1981; Jetz et al., 2001; Marples & Roper, 1996; Rowe & Guilford, 1996; Siddall & Marples, 2008, 2011). I found that ground beetles avoided infected hosts at all stages of infection based on the olfactory defence, providing protection for early stage infections perhaps while other defences have time to build up, such as the chemical defence.

With the olfactory defence eliciting such a strong response in ground beetles I further tested the generality of this defence using another potential predator of infected hosts, nocturnally foraging rodents, such as mice (Chapter 5). Utilising an olfactory behavioural assay I was able to test for any avoidance effects of infected hosts based on the olfactory cue. However, unlike the beetles (Chapter 3), house mice did not attend to the olfactory cue and showed no strict avoidance behaviours, only discriminating slightly at later stage infections, days 7 post-infection. Although a surprising result given that rodents have such a strong capability for scents, I decided to test the nocturnally foraging predators with the bioluminescent cue. Although not yet fully examined in this system (though it may be involved with maintaining the internal environment in infected hosts (Clarke, 2014)), there is evidence that bioluminescence has the capability to act as a warning signal in chemically defended prey (De Cock & Matthysen, 1999, 2003; Matthysen & De Cock, 2001). Utilising a similar behavioural assay, and knowing the olfactory cue does not elicit any avoidance behaviour, I tested whether mice demonstrated any avoidance behaviours towards glowing (nematode infected prey) or non-glowing (uninfected controls). I found that mice would use the light cue to avoid glowing

nematode infected prey, spending less time near infected hosts compared to uninfected hosts. Therefore although bioluminescence may be functional metabolically (Clarke, 2014), it might also be utilised as deterrent signal, protecting infected hosts from predation by nocturnally active predators.

Having examined a number of the defences I decided to test the olfactory and visual (colour change) cues in birds to determine which they attend to. Each defence has been studied in isolation to some degree with each seeming to confer some benefit to the nematode-bacterium system against a range of different predators. However, some predators attended to multiple cues, suggesting the defences employed may be acting in a multimodal fashion, where components of the signal occur in more than one sensory modality (Rowe, 1999; Scheffer, Uetz, & Stratton, 1996). Additionally, following evidence of vulnerability at early stages of infection (Chapter 3) the defences may be acting at various stages of attack to try to minimise the risk of predation (Endler, 1986, 1991). Utilising great tits as predators in a laboratory setting I decided to test both of these hypotheses. In terms of attack on infected individuals I found mixed effects to having a multiple cues, i.e. both the olfactory and visual (colour change) as it was not necessarily reducing attacks to the same extent as either cue singly. Additionally, there was evidence that scent was overshadowing the colour cue at various stages of infection. Furthermore, when determining the effects of multimodality specifically, we found no benefit in terms of initial attack on prey, although this should be tested more rigorously and does not rule out the possibility of multimodality within this system (see *future work)*.

Collectively, this thesis demonstrates an adaptive value to the various defences utilised in this system as well as providing the first evidence of the roles of

some defences, such as bioluminescence. However, producing each of the defences is likely to be costly and so each must provide some sort of benefit to be maintained within the system. The visual cue (red colour change) acts against predators with a good visual system, such as birds (Chapters 2 and 4). Additionally, bioluminescence acts to deter nocturnally foraging mammals, in this case, mice (Chapter 5). The olfactory cue was also seen to deter predation of infected hosts by beetles (Chapter 3), birds (Chapter 4) and to some degree mice (Chapter 5).

This thesis proposes that these defences have a protective role in reducing attacks and consumption of infected hosts which would ultimately result in parasite death. There is evidence however that the defences within this system may exist as a by-product of metabolism, as has been suggested for bioluminescence (Clarke, 2014). It is therefore unknown whether these parasite manipulations are targeted for defence or have been subverted as protective defences against predation. In this vein, it is not known whether these defences are exaggerated above the level produced metabolically or whether these metabolic products simply provide an additional benefit. As parasites kill their host, the bacterium replaces these defences with its own (colour change, chemical defence, olfactory defence) and it could be hypothesised that the bacteria may exaggerate these traits if they increase survival of the parasitic colony. These defences therefore may have become more exaggerated over time and in the future may evolve to become even more so if they confer some benefit to the parasite. It is however difficult to distinguish between an advantageous by-product and an advantageous direct product of selection (Poulin, 2010). However, you would expect selection to favour parasites that would induce more pronounced changes in their host depending on the cost-benefit relationship, if this resulted in reduced predation and survival of the parasite, as in this system.

I demonstrate that different predators attend to different defences within this system, in keeping with the idea that the defences operate in a multimodal fashion. This thesis adds to the growing field demonstrating multimodality in a warning signal context (Marples & Roper, 1996; Marples, Van Veelen, & Brakefield, 1994; Siddall & Marples, 2008) as much attention has been given to multimodality in a sexual signalling context (Hebets & Uetz, 1999; Scheffer et al., 1996). Though there are a large number of hypotheses as to the evolution of multimodality within systems (see Rowe & Halpin, 2013), there are a number which may be applicable to this system. Multimodal signals have been proposed to act in a sequential manner due to the unique properties of different sensory modalities that make them more detectable at different distances or environmental conditions (Candolin, 2003; Hebets & Papaj, 2005). The work in this thesis supports this hypothesis as it has highlighted the vulnerability of infections early on during infection whilst other defences build up. Therefore, it is plausible to suggest that multiple defences exist within this system as the defences are generated, and to some extent, deployed in a sequential manner.

Furthermore, it has been hypothesised that multimodality is beneficial owing to the perceptual variability in predators that rely on signal components in different sensory modalities (Rowe & Halpin, 2013). This thesis provides evidence of this as various predators did indeed attend to different cues within this system. As mentioned previously, birds would utilise both the visual and olfactory cues to varying extents when assessing and attacking prey. Beetles, however, with their poor visual systems, prioritised the olfactory cue, although this was not the case with mice, which utilised the bioluminescence cue to avoid infected hosts. Having multiple signals working in a multimodal fashion targeting different predators therefore helps

to minimise the risk of predation and ensure that there will be at least one cue present that a predator will be able to attend to.

Multimodality within parasite manipulations is now also increasingly recognised as parasites are capable of altering a number of phenotypic traits in their hosts, as in this system, rather than the traditional view of altering a single phenotype (Hughes, Brodeur, & Thomas, 2012; Thomas, Poulin, & Brodeur, 2010). Similarly to hypotheses on multimodality as discussed above, multidimensional manipulations (if there are at least two changes in different phenotypic traits) can occur either simultaneously or sequentially and can serve to increase transmission, though in this system the multidimensional nature of the parasite serves to reduce predation risk. So far, relatively few studies have considered the multidimensional nature of parasite manipulation, even fewer consider the ecological context of such manipulations (Thomas, Adamo, & Moore, 2005). This thesis therefore helps to bridge this gap in knowledge by examining the behavioural ecology of the multidimensional parasite *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*.

In conclusion, parasite manipulation through 'parasite-induced aposematism' is a novel and intriguing form of predator deterrence produced by an endosymbiotic bacterium in concert with its mutualistic nematode partner. Through acting in a multimodal fashion due to the multidimensional nature of the parasite and the number of potential predators, this nematode-bacterium system reduces predation risk and in doing so protects the parasitic colony within the host. In this way, *H. bacteriophora* and its symbiont *P. luminescens* could be viewed as signallers sending multiple messages to other individuals or species in order to elicit a

response, in this case, reduce predation. In addition to advancing the fields of host manipulation (through elucidating the roles of various defences in a novel system) and aposematism (by examining a novel form of 'infectious aposematism'), this thesis should trigger further research into this complex and intriguing system.

6.2 Future work

Although I have suggested some areas for future research in this area in the discussion of various chapters, I will briefly highlight the areas where I feel further work would advance the understanding of this system.

First and foremost I feel this system would benefit by examining the associated costs of carrying each of the defences within this system. GM (genetically modified) knockouts exist whereby each of the various defences (such as colour and bioluminescence) have been removed (D. Clarke, personal communication). This would allow comparison as to the costs of carrying each of the defences which could be easily carried out via competition assays in laboratory organisms. This could also be important in understanding the evolution of each of the defences as I would theorise that the cheapest defences may have arisen first in the system, followed by those that were more costly. This is an intriguing potential area for future research. Additionally, by producing bacteria without various defences it would be easier to test each defence in isolation with different predators. Furthermore, removing each defence may also alter the mutualism between nematode and bacterium as genes for each defence may be implicated in the bacterium-nematode mutualism. For example, the genes encoding anthraquinone production play an important role in

both the development of the red colouration of infected hosts and in toxicity, although they are not implicated in the association between nematode and bacteria (Brachmann et al., 2007). Although not implicated in the mutualism, the same cluster of genes are responsible for encoding various aspects of defence, meaning that if they were removed they could be detrimental for the survival of the parasitic colony.

This leads onto another potential area of further research examining the relationship between the nematode and bacterium. The bacterium produces changes in the host, with the nematode simply acting as a vector to transfer the bacterium between hosts. It is therefore possible to suggest that the bacterium may be capable of being free-living, away from its nematode host, given the appropriate conditions. Therefore further research examining the relationship between nematode and bacterium may highlight whether there is a co-evolutionary arms race between the two with the nematode constraining the bacterium and forcing it into the mutualism.

Wild type nematodes also exist with bacteria which produce defences to varying degrees, for example causing various levels of bioluminescence in infected hosts. It would therefore be interesting to see whether this correlates with levels of predation, i.e. would a higher level of bioluminescence deter nocturnally foraging mammals to a greater extent? This could therefore raise the question as to whether bacteria can alter the strengths of their defences based on either predator selection or availability of hosts. For example, in areas where avian predators are common, infected hosts might invest more in the visual defences which is the cue birds seemed to attend to most.

In conclusion, there are many areas of potential research which would hugely benefit this system which has only started to be examined recently. Although this

thesis provides some of the first evidence as to the roles of some the defences in this system, there is a large potential for future research and growth in this system which can be applicable to a wide number of fields, such as microbiology, host-parasite interactions and behavioural ecology.

6.3 References

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