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Investment in multiple defences protects a nematode–bacterium symbiosis from predation



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The act of predation often comprises multiple sequential steps whereby 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 *Photobacterium luminescens*. This nematode–bacterium complex infects and kills soil-dwelling insect larvae, 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 cues conferred a benefit to the infecting nematode–bacterium complex, utilizing feeding trials of nematode-infected waxworms, *Galleria mellonella*, with wild-caught great tits, *Parus major*. We tested for multimodality, as the cues 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. We then examined the first five prey attacked and 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.

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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-predator defences employed between species (Caro, 2005) and within species (van Buskirk, 2001). One reason for this is that individuals face attack from many predatory species; 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 utilize 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 proposed 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 more 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, Sherratt, and Speed (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 invest across different defences. Broom, Higginson, and Ruxton (2010) utilized 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, Broom et al. (2010) predicted investment across both defences. Furthermore, investment in multiple defences at different stages of predation are predicted when defences are relatively cheap or the individual has

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more resources available for investment in defence (Wang, Ruxton, Speed, & Broom, 2017). Additionally, investment in multiple defences has implications for the evolution of both predator and prey, as successful attack of a predator on prey depends on the number of defences for each species (Gilman, Nuismier, & Jhwueng, 2012).

Although a number of studies have examined multiple defences (van Buskirk, 2001; Jongepier, Kleeberg, Job, & Foitzik, 2014), these are normally considered in the context of multiple predators (Maleck & Dietrich, 1999; Poitrineau, Brown, & Hochberg, 2003; Rigby & Jokela, 2000; Sih, Englund, & Wooster, 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 the literature on 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 h, although, rather than decaying (Milstead, 1979), they undergo a number of changes. The symbiotic bacteria must then 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 synthesized by *P. luminescens* (Clarke, 2008). Hence the repellent odour is not that of a decaying corpse but something conferred by the nematode and/or its symbiont. Nematodes reproduce within this changing host and emerge 10–14 days postinfection before repeating the cycle of infecting a new host by cruising through the soil (Johnigk & Ehlers, 1999). Hence, predation at any stage will kill both the nematode and the symbiotic bacterium. 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 chemicals produced by *P. luminescens* deterred ants from feeding on waxworms infected with this bacterium (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 ones (Fenton et al., 2011). This effect was only seen in prey 5 or 7 days postinfection whereas at day 3 postinfection avian predators were equally likely to select an infected or uninfected waxworm. Furthermore, Foltan and Puza (2009) found that a related nematode species, *Steinernema affine*, deterred beetles from eating infected waxworms. Jones, Fenton, & Speed (2016) have recently reported an olfactory 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, Clarke, Fenton, Speed, and Hurst (2017) 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 deterrents (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.

METHODS

Experiments were run at the Konnevesi Research Station, University of Jyväskylä, Central Finland from January to 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). We examined multimodality first by examining the first attack in each experiment and then carried out a broader analysis to see whether it was consistent, even when subsequent behaviours were different.

Nematode Culturing

Waxworm larvae (Livefoods Direct, Sheffield, U.K.) were infected with the nematode strain *H. bacteriophora* TT01 (supplied by D. Clarke & S. Joyce, University College Cork, Ireland) by infecting 10 waxworms per petri dish containing 90 mm filter paper with 1000 infective juveniles/ml stock nematode solution. These were then frozen or utilized fresh depending on each of the three experiments.

Bird Housing

Ninety wild great tits were trapped at feeding sites at Konnevesi Research Station and ringed. Birds were kept in individually illuminated, ventilated plywood cages (64 × 46 cm and 77 cm high) indoors in a daily light period of 11.5 h. Sunflower seeds, feed balls (lard and seeds) and fresh water were available ad libitum except for 2 h 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 (50 × 50 cm and 57 cm high) that contained a perch and fresh water bowl. Birds were allowed to habituate to the experimental cage for at least 1 h 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 they were less aware of an observer. Owing to a lack of birds towards the end of the season, some birds ($N = 7$) participated in multiple trials but 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 used 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 signals. 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 four prey (one infected set and one uninfected) in or on petri dishes depending on the experiment. Infected prey were presented on one of days 3, 5 or 7 postinfection; uninfected prey were killed by freezing on the day of the trials. The uninfected waxworms were fresh and so will have shown no effects of decay, compared with infected individuals. Fenton et al. (2011) showed that wild robins, *Erithacus rubecula*, were significantly less likely to attack and consume *H. bacteriophora*-infected waxworms than uninfected controls, regardless of the age of the latter (i.e. either fresh or decayed for the same amount of time as the 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, we used four of each prey type (infected or uninfected) as birds were seen to attack eight 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 to give three conditions: (1) scent only, (2) colour only and (3) colour and scent together. We used 30 birds per experiment, 10 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 or day 3, 5 or 7 post-infection) under an 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. The visual stimulus was thus the same, but the odours (infected versus uninfected) could differ. To maximize the odour concentration, the petri dish was sealed with the lid and left for 1 h 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 the odours to escape.

Of the 30 birds used in the trial, half received infected waxworms on their left (six females, nine males) and half received infected waxworms on their right (seven females, eight males). The birds were observed for 20 min after the first attack on either prey and the order of prey taken, the number of prey consumed and any rejection behaviour (throwing or dropping prey) were recorded.

Condition 2: Colour Only

Four infected or 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 (five females, 10 males), and half on the right (six females, nine males). Birds were

observed for 20 min following an attack on either type of waxworm. Attacks were counted as pecks on the petri dish lid and approaches as the bird landing 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 1 h to allow the scent from both fresh infected and uninfected waxworms to diffuse. Birds were then observed for 20 min after initial attack and we recorded the number of waxworms attacked, consumed and rejected, as well as approaches to each dish. Of the 30 birds used in the trial, half received infected waxworms on the left (seven females, eight males) and half on the right (six females, nine males).

Statistical Analysis

To test for multimodality, we examined the first attack during each trial when the bird was naïve as each experiment provided a different feedback following attack on infected prey. We then examined the first five prey attacked and 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.

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 3×2 contingency table (Infection stage \times 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 test to examine the difference in the numbers of infected and uninfected waxworms attacked. We hypothesized 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 9×2 contingency table (Infection stage per experiment \times Infection) to examine whether there were any differences in the first waxworm attacked across each infection stage across each condition.

Attack rate

We coded whether a prey was among the first four (50%) attacked and how many of these were uninfected or infected prey, then ran a binomial generalized linear model (GLM) 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 used 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 participated 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 first examined probability of consumption per se and then 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 that they were attacked.

RESULTS

We discuss the results in three ways: first, attack rates on infected and uninfected prey across all three conditions; second, consumption of infected individuals during the scent only and colour and scent conditions and, third, as a multimodal signal. Although we examined multimodality using the first attack only, the results for the first five prey attacks must be interpreted with caution due to the experimental design.

Attack Rate

For the first four prey attacked, compared to the colour and scent condition, there was no significant difference in prey attacked based on condition (Fig. 1; scent only: $z_{240} = -0.897$, $P = 0.370$; colour only: $z_{240} = 0.230$, $P = 0.819$) or presence of infection (Fig. 1; $z_{360} = -1.78$, $P = 0.076$) for day 3 postinfection 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).

However, for day 5 postinfection prey there was a significant difference between the attack rates in the colour and scent versus the colour only condition (Fig. 2; $z_{120} = -2.426$, $P = 0.015$) but not versus the scent only condition (Fig. 2; $z_{120} = -1.350$, $P = 0.177$) and whether prey were infected or not (Fig. 2; $z_{360} = -5.712$, $P < 0.001$). Additionally, there was a two-way interaction between scent only and presence of infection ($z_{120} = 2.178$, $P = 0.029$) and between colour only and presence of infection ($z_{120} = 3.360$, $P < 0.001$). Therefore, at this stage (5 days postinfection), scent by itself is as effective a signal to deter attacks on infected individuals as colour and scent in concert.

For day 7 postinfection, there was a significant difference in attack rate between colour and scent versus scent only (Fig. 3; $z_{240} = 2.012$, $P = 0.044$) and whether prey were infected or not (Fig. 3; $z_{360} = -4.618$, $P < 0.001$). Additionally, there was an interaction between scent only and infection ($z_{120} = -2.581$, $P = 0.004$). Therefore, at this stage, scent by itself provided the best protection in terms of reduced attacks on infected individuals, but colour by itself was as protective as both signals together.

Consumption Rate

Significantly fewer infected waxworms were consumed in the colour and scent condition than in the scent only condition at day 3 postinfection (Fig. 4; $z_{80} = -2.622$, $P = 0.009$). 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; $z_{80} = 0.0$, $P = 1$) postinfection in either condition. Therefore, having colour and scent was beneficial for infected prey on day 3 postinfection, but scent alone provided as good a cue at days 5 and 7 postinfection.

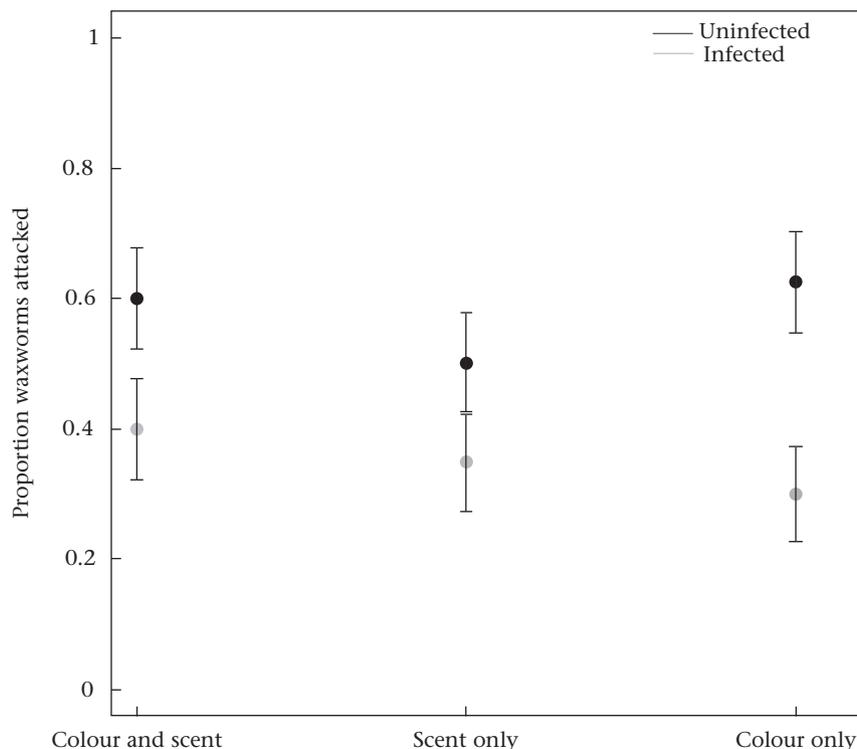


Figure 1. Proportion of uninfected or *H. bacteriophora*-infected day 3 postinfection waxworms attacked in the first four attacks across the three conditions (colour and scent, scent only and colour only). Bars represent SEs.

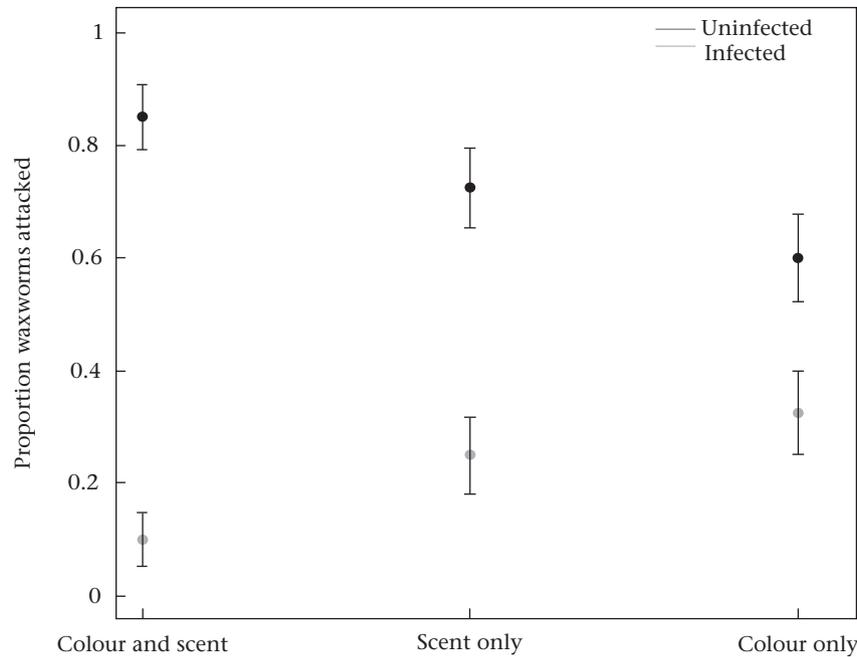


Figure 2. Proportion of uninfected or *H. bacteriophora*-infected day 5 postinfection waxworms attacked in the first four attacks across the three conditions (colour and scent, scent only and colour only). Bars represent SEs.

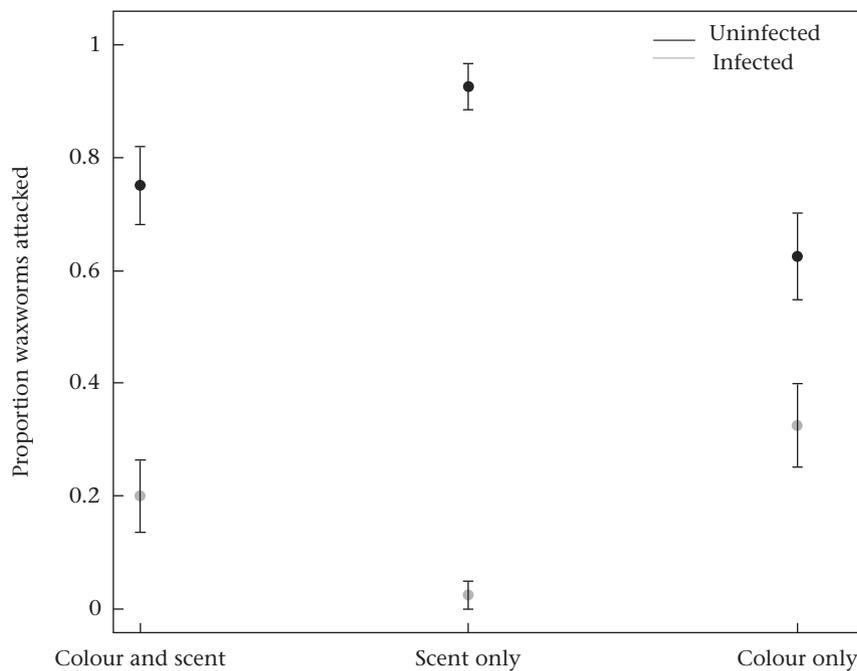


Figure 3. Proportion of uninfected or *H. bacteriophora*-infected day 7 postinfection waxworms attacked in the first four attacks across the three conditions (colour and scent, scent only and colour only). Bars represent SEs.

Additionally, following an attack, significantly fewer infected waxworms were consumed in the colour and scent than in 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 at 5 days postinfection ($z_{70} = -2.903$, $P = 0.004$). However, it is hard to interpret these interactions as on some days there were very few attacks on infected prey.

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

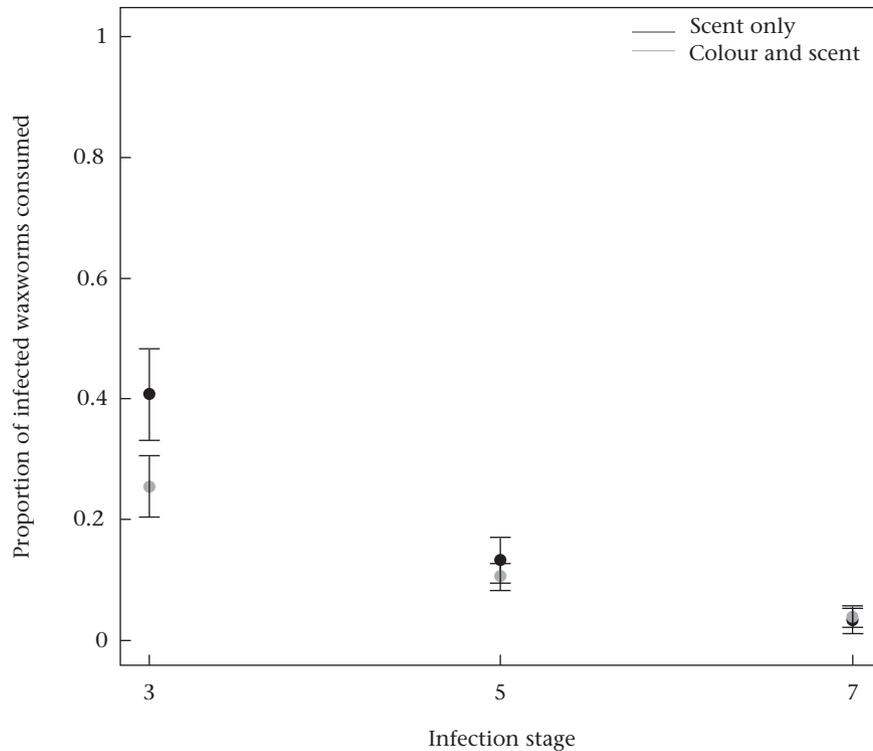


Figure 4. Proportion of *H. bacteriophora*-infected waxworms consumed in the colour and scent and scent only conditions across all three infection stages (3, 5 and 7 days post-infection). Bars represent SEs.

only (Table 1; $P = 0.192$), colour only (Table 1; $P = 0.893$), scent and colour (Table 1; $P = 0.249$) or across all three conditions (Table 1; $P = 0.306$).

Significantly more uninfected than infected waxworms were attacked first by naïve birds on day 7 postinfection during the scent only condition ($\chi^2_1 = 6.4$, $P = 0.011$). On day 5 postinfection, slightly more uninfected waxworms were attacked first ($\chi^2_1 = 3.6$, $P = 0.058$) and on day 3 postinfection there was no difference between infected and uninfected waxworms ($\chi^2_1 = 1.6$, $P = 0.206$). Therefore, although there was no overall effect of having either a unimodal or multiple defence, there appeared to be a benefit of scent for infected waxworms on day 7 postinfection.

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 more than either signal alone. In terms of attack rate on infected individuals, there was no benefit of multiple signals at day 3 postinfection but at day 5 postinfection, colour provided less protection than either colour and scent together or scent alone. However, at day 7 postinfection scent alone provided the best protection. Therefore, utilizing both colour and smell provided

protection at different stages of infection when birds attended to the different signals. However, our study contradicts others in which colour was a more salient cue than scent (Marples, van Veelen, & Brakefield, 1994) although this could just be an artefact of experimental design as birds had different reward experiences once they had attacked prey. For example, birds were not able to feed in the colour only trial and so had no gustatory feedback. In seven-spot ladybirds, *Coccinella septempunctata*, colour pattern was the most important cue, followed by taste (Marples et al., 1994).

This phenomenon, known as ‘overshadowing’, 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; Roper & Marples, 1997; Rowe, 1999). This can also prevent the predator from learning one signal in the presence of another (Siddall & Marples, 2008). Couvillon and Bitterman (1988) found that colour was overshadowed by odour during a 10 min extinction test following presentation of colour–odour combinations to honeybees, *Apis mellifera*. In this study, we found similar effects of scent overshadowing colour in terms of attack at late stages of infection (day 7 postinfection). Colour, on the other hand, did not seem to provide much protection, unless in combination with scent.

Table 1
Number of uninfected or *H. bacteriophora*-infected waxworms attacked first for each infection stage (3, 5 and 7 days postinfection) 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

Furthermore, scent and colour only appeared 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 was present. However, later during infection, scent by itself provided as much protection as colour and scent in concert, suggesting that the scent signal was then overshadowing the colour signal. Therefore, although in this case colour did not seem to confer a benefit, in combination with scent it could minimize predation and consumption and thus both defences are maintained within this system.

Although in this study both colour and odour were considered in the identification stage of defence, a relatively early stage of the predation sequence, they both conferred benefits to the infected individual through reduced attack. Although the costs of each defence are currently unknown, having both defences present at an early stage may mean each is relatively cheap to produce or that the nematode–bacterium complex has more resources available for investment in defence (Wang et al., 2017). However, investment in multiple defences, in this case colour and odour, 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 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 the system we studied, ground-foraging invertebrate and mammalian predators are likely to encounter infected hosts which are likely to prioritize different defences based on the predator's perceptual capabilities, such as invertebrates attending to olfactory signals (Jones et al., 2016) or other chemical signals (Gulcu et al., 2012; Zhou, Kaya, & Goodrich-Blair, 2002) and mice attending to bioluminescent signals (Jones et al., 2017).

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). As well as colour and scent, infected hosts also bioluminesce (Ffrench-Constant & Bowen, 2000) and have a chemical defence (Baur et al., 1998; Gulcu et al., 2012; Zhou et al., 2002). Therefore, one can hypothesize 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 postinfection individuals in the scent only condition whereby more uninfected than infected individuals were attacked. However, owing to the nature of our experiments, it would be intriguing to test each defence (taste, colour and scent) in a factorial design to fully elucidate whether this system is acting in a multimodal signalling manner.

Many studies have examined how odour and/or sound interact with warning coloration to deter predation in domestic chicks, *Gallus gallus domesticus*, utilizing 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 and Marples (2011) found that wild robins learnt to avoid yellow baits treated with artificial pyrazine (a common insect warning odour) faster than those with no odour. However, it is 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). They presented seven-spot

ladybirds to captive Japanese quail, *Coturnix coturnix japonica*, in treatment combinations with colour pattern, scent and taste singly, in a two-way combination or the whole insect. Avoidance was maximized 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 Rowe & Halpin, 2013). Some relate to how multiple signals can increase the 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 the 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 have considered these defences in tandem. The defences in this nematode–bacterium system occur across multiple stages of predation and we found colour and scent by themselves were as beneficial as both signals together at various 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 symbiotic system. 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 predators with different perceptual capabilities at various stages of predation.

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