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The influence of multimodal warning displays on avian avoidance behaviour.

Emma Caroline Siddall

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy to the University of Dublin, Trinity College.

Department of Zoology
April 2008
Declaration

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Emma C. Siddall
Summary

This thesis empirically investigates the effects of insect multimodal warning displays on avian predatory decisions. Specifically, it examines the effects of pyrazine odour, and the agitated buzzing of a bumble bee on innate and learned avoidance behaviour of birds towards yellow and red prey, and the memory of these learned avoidances. Domestic chicks were used as the experimental subjects for the laboratory experiments, and European robins for the field experiment.

Four measures of innate avoidance behaviour were compared: latency measures including neophobia and dietary conservatism, the number of crumbs of each colour eaten, and a derived measure, eating bias. Pyrazine odour was observed to prolong both neophobia and dietary conservatism of yellow and red crumbs. It was found that the colour signal needed to be novel to the chicks for innate avoidance to be observed, but familiarity with the odour signal did not reduce innate avoidance. Buzzing was recorded to have no effect on any measure of innate avoidance behaviour. When the four measures of innate avoidance were compared it was concluded that dietary conservatism offered the most reliable measure of avoidance. Neophobia appeared to be a quick response that was very variable. The eating bias measure often gave misleading results, and therefore the results from this derived measure must be interpreted using number of crumbs of each colour eaten in order to get an accurate view of the behavioural response.

Pyrazine enhanced avoidance learning of both yellow and red prey. The odour also prolonged memory of the yellow prey, and it was noted that both components of the multimodal display needed to be present in order for the learned avoidance to be recalled. The implications of this result for mimicry are discussed. The pyrazine odour
reduced the generalisation of the learned avoidance from the unpalatable yellow to the palatable green crumbs, allowing the birds to learn to avoid the unpalatable prey while continuing to exploit the palatable prey. As in the case of innate avoidance behaviour, buzzing had no observable effect on avoidance learning or memory. A comparison between a laboratory experiment using domestic chicks and a field experiment using European wild robins showed that domestic chicks and wild birds respond to pyrazine in a similar manner, and that pyrazine enhanced avoidance behaviour in both groups.

The implications of these results are discussed in the context of the evolution of insect warning displays, and the psychological issue of whether the component cues of multimodal signals operate as alerting signals or warning signals in their own right is addressed.
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CHAPTER 1 – GENERAL INTRODUCTION
1.1 – INTRODUCTION

Several areas need to be considered in order to introduce the research questions addressed by this thesis. Section 1.2 discusses aposematic warning signals in general. In order to fully appreciate how signals through different sensory modalities affect avian predatory decisions, it is necessary to have an understanding of the major senses in birds; consequently Section 1.3 examines visual, olfactory and auditory perception in birds. Section 1.3 also discusses the body of research investigating the effects of visual, olfactory and auditory warning cues on avian predator responses, and their presentation as multimodal signals. Guilford and Dawkins (1991) argued that receiver psychology is important in shaping the evolution of warning displays; therefore, in order to understand the design of these signals it is necessary to review predator responses to aposematic species, addressed in Section 1.4. Section 1.5 considers how predator responses might convey protection to mimetic species, and discusses some implications of multimodal warning displays on mimicry. Domestic chicks (*Gallus gallus domesticus*) have frequently been used as model avian predators in research in this area, and are the experimental subjects throughout most of this project. Section 1.6 provides a justification for why chicks may be good model predators, but also highlights some of the limitations of this model species. This section also discusses the use of artificial prey in experimental studies. Finally, Section 1.7 outlines the research questions investigated in this thesis.
1.2 – APOSEMATISM

Many prey species have adaptations which make them less profitable to potential predators. These adaptations include increased handling time and/or chase time, higher probability of escape, or toxicity, either through toxins produced internally or sequestered from the diet (Guilford 1990; Schuler and Roper 1992). Defended prey species frequently advertise their defended state using warning signals (Ruxton et al. 2004). Much research has examined how prey communicate unprofitability through visual conspicuousness (Gittleman et al. 1980; Roper and Wistow 1986) using warning colours such as red and yellow (Sillén-Tullberg 1985a; Roper 1990; Schuler and Roper 1992; Ingalls 1993) and certain patterns (Schuler and Hesse 1985; Roper and Cook 1989). Recent work suggests that other modes of signalling, such as olfactory and auditory cues, may also be of importance in conveying unprofitability to potential predators (Marples et al. 1994; Rowe and Guilford 1996, 1999a, b; Rowe 1999; Skelhorn and Rowe 2005). There is extensive similarity across many taxa within the visual (e.g. colour) and olfactory (e.g. pyrazine) warning signals used by defended species (Rothschild and Moore 1987; Moore et al. 1990). It has been suggested that these similarities may represent a case of global Müllerian mimicry in which all defended species share a common set of warning signals towards which predators behave in a wary manner (Sherratt 2002).
1.3 – PREDATOR SENSES AND INSECT WARNING SIGNALS

In order to appreciate how avian predators respond to the warning displays of their insect prey, it is necessary to understand their sensory capabilities. This section seeks to summarise the relevant senses in birds and to discuss the insect warning signals that occur through various sensory pathways.

1.3.1 – Avian vision and visual warning signals

a) Avian vision

Vision is the dominant sense in birds (Linzey 2001), and diurnal birds may have one of the most complex forms of vertebrate colour vision (Bowmaker 2004). The bright sexual displays common across many groups of birds suggests that they must have excellent colour vision (Bowmaker 2004). Human vision is trichromatic, with a range between 400nm in the violet wavelengths and 750nm in the red wavelengths (Dartnall et al. 1983). Many animals, including birds, see beyond this range. Birds have tetrachromal vision, possessing four spectrally distinct classes of cones: red, green, blue and UV (Goldsmith 1980). Oil droplets are also present in the avian eye; these droplets serve to filter out short wavelengths of light before it reaches the cones (Church et al. 2004). This increases the sensitivity of each cone type and enhances colour discrimination (Dyer 2001).

Most bird species can detect light within the Ultraviolet range, 300 – 400nm (Bennett and Cuthill 1994). Ultraviolet vision in birds may mean that they perceive many visual signals differently to humans, which may affect how they interact with potential prey species (Church et al. 1998). Colour vision in the chicken is similar to
that of other diurnal birds, making them a good model for the study of avian vision (Bowmaker 2004).

Although birds can see different parts of the visual spectrum to humans, a study by Jones et al. (2001) suggests that they still categorise colours in a similar manner to humans, and generalisation occurs in a similar direction. However, Osorio et al. (1999) showed that birds can discriminate between colours that humans find it difficult to discriminate, which is probably due to the presence of oil droplets as discussed above.

b) Visual warning signals

The correlation between distinctive visual displays and defence mechanisms in many prey species was first noted by Wallace (1867). He suggested that these visual warning signals may reduce the number of individuals sacrificed to educate the predator, and prolong protection once predators have been educated (Darwin 1871). Poulton (1890) coined the term “aposematic colouration”, which referred to these visual signals that advertise an individual’s defended state to potential predators. Because humans are visual species (Rock and Victor 1964), the majority of research into insect warning displays has concentrated on how aposematic insects advertise their defended state using visual signals (Schuler and Roper 1992). There is much evidence to support Wallace’s suggestion that warning colouration enhances protection from predation. Indeed, visual signals which are conspicuous rather than cryptic (Gittleman and Harvey 1980; Roper and Redston 1987; Roper 1994), novel rather than familiar (Coppinger 1970; Shettleworth 1972; Roper 1993), and warningly coloured such as red (Sillen-Tullberg 1985a, b), yellow, orange and white (Guilford and Dawkins 1991) have all been observed to increase the rate of avoidance learning. Gamberale-Stille and Guilford (2003) suggest that colour may be more important than contrast or patterns in
avoidance learning. Features of visual signals which enhance learning, such as conspicuousness, may also prolong the memory of the learned avoidance (Roper and Redston 1987); however, this experiment used methyl anthranilate to make the prey unpalatable, and this substance has been shown to have an odour to which chicks respond (Marple and Roper 1997). The faster learning rates may therefore not have been due solely to the visual conspicuousness of the prey (see Section 1.3.4).

There is also evidence that birds have an unlearned wariness of certain warning colours and patterns, such as black and yellow striped prey (Schuler and Hesse 1985; Roper and Cook 1989) and red prey (Coppinger 1970; Roper 1990; Mastrota and Mench 1996). Roper and Cook (1989) provided evidence that a striped pattern is important in eliciting innate avoidance, since if the prey was coloured half yellow and half black, no avoidance was recorded. Novelty of the visual signal is also important for eliciting innate avoidance behaviour (Roper 1990, 1993), and experience can turn off these unlearned aversions (Marple et al. 2007). Schuler and Roper (1992) noted that the innate wariness exhibited towards a visual signal is dependent on the context in which it is presented. Chicks showed an innate avoidance of red when it was presented with brown food (Roper 1990), but no such avoidance when red was presented with olive green food (Roper and Cook 1989). This is discussed further in Section 1.4.1a.
1.3.2 – Avian olfaction and olfactory warning signals

a) Avian olfaction

Mammals have far better olfactory capabilities than birds (Roper 1999). Due to the placement of the nares on the posterior dorsal surface of the beak, and because birds do not exhibit scent marking behaviours or obvious intraspecific olfactory communication, it has long been believed that olfaction is not an important sensory mode in birds (Roper 1999), and that birds have a poorly developed sense of smell (Järvi and Wiklund 1984; Jones 1987). However, neurophysiological and behavioural evidence has shown that many bird species possess good olfactory capabilities (Jones and Roper 1997). Birds may use olfaction for navigation, as in the case of the homing pigeon (Walraff and Andreae 2000; Clark and Mason 2000), or for foraging, as in New World vultures (Gomez et al. 1994; Nevitt et al. 1995), petrels and albatrosses (Nevitt et al. 2004). There is also evidence that birds use olfaction to choose nesting materials with biocidal properties (Clark and Mason 1987; Clark and Smeraski 1990) and for avoiding toxic insects (Rothschild and Moore 1987; Rowe and Guilford 1996, 1999a, b; Roper and Marples 1997a). It would seem that although vision is the dominant sense in birds, olfaction is used when vision cannot provide sufficient information (Healy and Guilford 1990; Bonadonna et al. 2001; Evans and Hesier 2001).

Domestic chicks can learn to recognise specific olfactory cues associated with food items and can then use these cues to optimise their intake of appropriate food (Turro et al. 1994). Further, odour can be used as a discriminatory learning cue in the absence of a visual signal (Guilford et al. 1987), and in some cases odour may overshadow learning about a visual signal (Roper and Marples 1997a). Chicks are considered to be capable of olfaction the day prior to hatching (Romanoff 1960;
Tolhurst and Vince 1976), which would suggest that day-old chicks are a good model for avian olfaction studies.

b) Olfactory warning signals

Defended prey species often emit a distinctive nasty odour when threatened (Wallace 1891; Rothschild 1961; Majerus 1994). Pyrazines are common warning odours found in a diverse range of aposematic insects across many taxa and geographical locations (Cott 1940; Rothschild 1961; Rothschild et al. 1984; Moore et al. 1990; Woolfson and Rothschild 1990). Rothschild and Moore (1987) suggested that pyrazines may act as alerting signals, drawing the predator’s attention to the food and therefore aiding differentiation of the signal from its background, making it more conspicuous.

The inclusion of pyrazine odour in an insect’s warning display may provide it with additional protection from predation. Pyrazine odour has been shown to elicit unlearned biases against conspicuous (Lindström et al. 2000), novel (Jetz et al. 2001) and warningly coloured prey (Marple and Roper 1996; Rowe and Guilford 1996, 1999a). Rowe and Guilford (1996) noted that pyrazine odour could elicit a bias against yellow food, when none was observed in the absence of the odour. Further, Marple and Roper (1996) tested the effects of five novel odours and noted that only odours typically associated with warning displays (pyrazine and almond) enhanced innate avoidance behaviour. Notably, certain palatable species mimic the olfactory warning displays of defended insects, but are not themselves toxic (Moore et al. 1990), which demonstrates that pyrazine acts as a signal rather than an extra aversive substance.

Marple and Roper (1996) argued that visual cues need to be novel in order for odour to elicit innate avoidance. However, Rowe and Guilford (1999a) observed that pyrazine could elicit avoidance of familiar warning coloured food. Even though the
chicks in the Rowe and Guilford (1999a) study were familiar with the yellow crumbs, they showed avoidance towards them in the presence of the novel pyrazine odour. In their study, Marples and Roper (1996) used colours not typically associated with warning displays, whereas Rowe and Guilford (1999a) utilised classical warning signals, which may explain the disparity between these results. However, there is a continuing debate in this area, and further research is needed to clarify this point.

Once the predator has overcome its initial neophobia and started to sample the novel prey, olfactory warning cues may also enhance avoidance learning. Pyrazine can act as a discriminatory cue for learned avoidance in the absence of visual cues, as has been observed for both chicks (Guilford et al. 1987) and rats (Kaye et al. 1989). This suggests that pyrazine might act as a learning cue in its own right and not merely enhance, or potentiate, learned avoidance of warningly coloured prey, as previously proposed by Rothschild and Moore (1987). Barnea et al. (2004) demonstrated that pyrazine enhanced domestic chicks' ability to avoid distasteful water presented in a red tube. Roper and Marples (1997a) observed an enhancement of learned avoidance with two other non-warning odours, almond and vanilla. All four of these studies used liquid rather than solid model prey items. Roper and Marples (1997b) observed that the response towards solid and liquid prey often differed when the solid prey was presented on the floor of the experimental arena, while the liquid was presented in a drinker; the method used by Guilford et al. (1987), Kaye et al. (1987), Roper and Marples (1997), and Barnea et al. (2004). This discrepancy appears to be due to differences in the mode of presentation of the solid and liquid prey, as no differences were observed when both the solid and the liquid prey were presented in a Petri dish on the floor of the experimental arena (Roper and Marples 1997b). The effects of the modes of presentation on behavioural responses may be due to contrast of the prey with the background of the experimental arena. Given these observations it is necessary to
verify previous findings from liquid prey using solid prey, or liquid prey presented on the floor of the experimental arena.

Roper and Marples (1997a) suggest that odours may sometimes be more important signals for discrimination learning than visual cues. They provide evidence that almond odour is an almost equivalent learning cue to novel green or blue food. Almond odour may overshadow colour cues, such that water tainted with quinine and smelling of almond may be avoided by chicks even if it is a different colour from that with which almond odour was originally associated.

In their study of the memory-enhancing effects of a conspicuous visual signal, Roper and Redston (1987) made their prey unpalatable using methyl anthranilate. Methyl anthranilate has a noticeable odour (Marples and Roper 1997); therefore, Roper and Redston’s (1987) distasteful prey possessed both visual and olfactory warning signals although the importance of this was not recognized at the time nor mentioned in their paper. Their results show that unpalatable odorous-conspicuous prey were avoided more than unpalatable odorous-cryptic prey. One possible explanation for this result is that the odour cue may have interacted more with the conspicuous than the cryptic visual signal. Thus this experiment did not unambiguously demonstrate that the more conspicuous signal enhanced memory of the prey’s aversiveness.

Odour signals may also affect memory of learned avoidance behaviour. Guilford et al. (1987) trained chicks to avoid tainted water accompanied by pyrazine odour. After a 24-hour retention interval the chicks showed no avoidance of the water; however, they did show more signs of distress during the subsequent test than untrained chicks, suggesting some recollection of the meaning of the pyrazine odour. However, this study was conducted without a colour cue, and so represents the memorability of pyrazine as a lone signal. Barnea et al. (2004) noted that pyrazine odour prolonged memorability of unpalatable water in red, yellow and green tubes, but
again, the only evidence for memorability being enhanced by odour comes from experiments using liquids as prey.

1.3.3 – Avian hearing and auditory warning signals

a) Avian hearing

Hearing is keen in birds and second to sight in importance (Linzey 2001). Birds' hearing capabilities are acute, so there is no physiological reason why they should not detect and respond to auditory cues (Linzey 2001; Dooling 2004). However, in comparison to mammals, birds do not hear well at either high or low frequencies. There are no examples of birds hearing sounds above 15 Hz (Dooling 2002). Although some birds are specialised to hear a different range of frequencies, most birds have the greatest sensitivity of hearing between 1 kHz and 5 kHz with absolute sensitivity falling between 0 and 10 dB (Dooling 2004), while humans can hear between 20 Hz and 15 kHz.

Birds have good directional hearing (Larsen 2004), such that they can determine the source of a sound relatively accurately. This is an important ability, used to determine the location of potential mates, competitors and prey. As birds have small heads, the time between the sound hitting one ear and then the other is markedly shorter than in humans. Due to this, it has often been incorrectly assumed that birds had poor directional hearing. However, it is now accepted that birds use the interaural canal, the air-filled canal connecting their inner ears, to overcome this problem (Larsen 2004). The time lag between sound waves hitting the external part of the ear and the interaural canal is used to determine sound direction.

There are many examples of bird behaviour being altered by an auditory-visual multimodal signal. Visual imprinting in chicks may be improved when an auditory cue
is presented simultaneously with the visual stimulus (Brown 1975; Van Kampen and Bolhuis 1993). Hultsch et al. (1999) observed from laboratory tests that a flashing light increased the rate and extent of song learning in nightingales (*Luscinia megarhynchos*), and that a compound signal comprised of auditory and visual components is learned more effectively than either component cue alone. Further, young cuckoos (*Cuculus canorus*) need to mimic both the visual and vocal cues of reed warbler chicks (*Acrocephalus scirpaceus*) in order to fool reed warbler parents into caring for them (Kilner et al. 1999).

There is a greater level of similarity between the hearing capabilities of different bird species than amongst other vertebrate taxa (Dooling 2002). This suggests that chicks in the lab may well reflect the auditory behaviour of other bird species. Saunders and Salvi (1993) suggest that chicks are a good model for the study of hearing in general. Song learning occurs quite early in birds (Dooling 2004) and hearing develops neonatally in the domestic chick (Jones et al. 2006). Adult chickens appear to be 5 to 15 dB more sensitive than 4-day old chicks, such that as the chick ages it becomes more sensitive to a wider frequency of sounds (Saunders and Salvi 1993). This would suggest that observations made about hearing using chicks as a bird model may represent a conservative estimate of the behavioural responses of other bird species to auditory signals. If auditory cues have an effect on the behaviour of young chicks, then it is likely that the signal will have an effect on the behaviour of adult birds also.

**b) Auditory warning signals**

Very little research has been conducted on how auditory cues affect predatory decisions, despite the fact that researchers have anecdotally reported hearing a multitude of sounds produced by defended prey species (Rowe and Guilford 1999b).
Stridulation (Masters 1979), hissing (Bedford and Chinnick 1966; Kirchner and Roschard 1999), clicking (Dunning 1967; Brown et al. 2007) and buzzing (Rowe and Guilford 1999b; Hauglund et al. 2006) have all been associated with insect warning displays and may operate as warning sounds. Kirchner and Roschard (1999) noted that mice (Mus domesticus) avoided the hissing noise made by bumblebees (Bombus terrestris) when offered a choice test between a tunnel in which hissing was played and a silent tunnel.

To date there have been only three empirical investigations into the effect of auditory signals on avoidance behaviour in birds. Rowe and Guilford (1999b) noted that the agitated buzzing of B. terrestris enhanced innate avoidance towards novel green or yellow crumbs. They hypothesised that innate avoidance behaviour may be enhanced by any auditory stimulus as long as it is novel. However, Hauglund et al. (2006) conducted a similar experiment using the non-agitated buzzing of a flying wasp (Dolichovespula media) played back to the chicks at 65-72 dB, which is well above the absolute threshold of hearing for birds (Dooling 2004), and found no effect of buzzing on mean avoidance of novel yellow prey. This may reflect that agitated buzzing operates as a warning signal, whereas buzzing during flight does not.

Evidence for sounds altering the speed of avoidance learning is equally limited. Rowe (2002) examined how many trials chicks took to achieve discrimination between rewarded and unrewarded prey, and noted that an artificial beeping sound reduced the number of trials necessary; however, this study used visual and auditory cues not typically associated with warning displays, which may have had an effect on the results. The buzzing of D. media does not appear to affect learned avoidance behaviour in chicks (Hauglund et al. 2006). Hauglund et al. (2006) provide the only investigation into whether a warning sound can affect memorability of a learned avoidance, and noted that buzzing, if anything, appeared to speed up forgetting.
1.3.4- Multimodal warning signals

Vision is the dominant sense in human perception (Rock and Victor 1964), and this may have led to an underestimation of the importance of other sensory modalities in other species (Marples and Roper 1996). However, a warning display may often consist of several component cues through different sensory modes, thus creating a multimodal warning signal (Guilford and Dawkins 1991), which together constitute an "aposematic syndrome" (Schuler and Roper 1992). There is a growing body of evidence which suggests that many insect prey species may signal their unprofitability through non-visual as well as visual cues (Rowe 1999; Ruxton et al. 2004).

It has been debated whether cues through different sensory channels are signalling to the same or different predators (Rothschild 1965; Pearson 1989; Rowe 1999); recent research supports the former argument. Component signals in a multimodal display often operate synergistically, eliciting a greater response than the sum of the responses to the individual component signals. This suggests that the multiple cues are aimed at the same receiver (Marples et al. 1994; Marples and Roper 1996; Rowe and Guilford 1996, 1999a, 1999b; Rowe 2002; Siddall and Marples 2008). Further, multimodal displays may be a more reliable indicator of a prey's profitability than monomodal signals and could enhance innate and learned avoidance, and the memorability of encounters with unprofitable prey (Guilford and Dawkins 1991; Marples et al. 1994; Rowe 1999). As such, it is necessary to study multimodal signals holistically, as by studying signal components in isolation, the synergistic action of the multimodal display will be missed (Rowe 1999).

A multimodal display may be perceived as a compound signal independent of the component signals of which it is composed (Rowe 1999) due to synergistic interactions between component signals. In other words, the predator may respond differently to the colour, smell, taste and sound of the prey when all these cues are
present together than it would have if it had experienced each component individually. A combined cue may be considered a qualitatively different signal, not just a combination of its component parts (Kehoe and Graham 1988; Kehoe et al. 1994; Rowe 1999). If compound displays are perceived independently of their component signals, then contextual isolation may occur; prior experience with a component signal outside the multimodal context is disregarded when the cue is part of a compound display (Rowe and Guilford 1999b).

One intriguing finding in support of contextual isolation is that pyrazine can elicit avoidance of familiar warningly coloured (Rowe and Guilford 1999a, b) and conspicuous food (Lindström et al. 2000) which the predator has previously perceived as profitable. Contextual isolation is an integral part of the theory of multimodal signalling, and seems essential if experienced predators are to exhibit avoidance towards warning signals. If a predator is willing to eat all yellow coloured prey after experiencing profitable yellow food, then yellow will be rendered useless as a warning signal. However, if yellow prey that also had a distinctive odour were viewed as novel, then predators could learn to avoid the prey, and yellow would still be an important component of the warning display. Despite its importance in predator responses to warning signals, contextual isolation has received very limited empirical investigation.

1.4 - PREDATOR RESPONSES TO APOSEMATIC PREY

When a predator encounters a novel defended prey species, a succession of psychological processes occur. Initially the predator exhibits innate avoidance of the novel prey. Then, through experience, the predator learns to associate the prey's warning signals with its profitability. Upon meeting the prey species at a later date, the
predator may recall previous experiences with it and will choose to accept or reject the prey item depending on the profitability of those experiences.

These psychological responses are dependent on the signal received by the predator (Guilford and Dawkins 1991). Often a prey may signal through more than one sensory modality, creating a multimodal signal, as discussed in Section 1.3.4. Predator responses to aposematic prey will be discussed in more detail in the sections below.

1.4.1- Innate avoidance

a) Innate wariness

Predators tend to exhibit initial wariness when presented with novel prey. This hesitancy to attack novel food may cause a reduction in predation pressure on novel prey relative to familiar prey (Schuler and Roper 1992). Fear is often adaptive and functions to protect an animal from injury (Jones 1996), so that by exhibiting wariness predators decrease their chances of being poisoned by prey of which they have no knowledge. It is possible that predator biases, such as wariness towards novelty or certain colours, may have evolved due to the existence of common warning signals across many invertebrate prey species (Sherratt 2002). Innate avoidance is a heritable trait (Marples and Brakefield 1995); therefore, naïve chicks may express innate avoidance behaviour that they inherited from their parents. These biases may also be passed from one generation to another culturally (Lindström 1999). Doherty and Cowie (1994) demonstrated that canaries (*Serinus canaria*) showed extended fidelity to that food they were fed by their parents as chicks.
Both domesticated and wild naïve birds exhibit wariness towards novel (Roper 1993; Marples and Roper 1996; Roper and Marples 1997; Marples *et al.* 1998) and warningly coloured food (Schuler and Hesse 1985; Sillen-Tullberg 1985a; Roper 1990; Rowe and Guilford 1996). Innate wariness of novel food may afford defended prey species considerable protection from naïve predators (Marples and Kelly 1999; Speed 2000; Kelly 2001) in a similar mechanism to the function of startle responses (Schlenoff 1984). These phobic reactions are flexible; experience with a novel coloured food can deactivate wariness to other coloured foods as long as all prey items are palatable (Marples and Roper 1996; Marples *et al.* 2007).

Sillen-Tullberg (1985a) noted that red morphs of the larvae of *Lygaeus equestris* elicited a greater innate avoidance and faster learning rates than grey morphs whether they were presented on a red or a grey background. These results indicate that signals that promote innate avoidance may also promote learning, a suggestion which is supported by Lindström (1999). This may not be true for all signals. Signals which evoke innate wariness and those which are memorable may not always be the same, and a trade-off may occur between these two selective forces (Braveman and Jarvis 1978; Miller and Holzman 1981; Roper and Cook 1989). The possible existence of this trade-off has scarcely been addressed in the literature and warrants further investigation.

**b) The two distinct processes of innate avoidance behaviour**

There is often large variability in the wariness exhibited by individuals in a population towards novel prey (Marples *et al.* 1998). Despite this, most of the past research on the subject has treated it as a uniform response (Sillen-Tullberg 1985a; Rowe and Guilford 1996, 1999a, b; Skelhorn and Rowe 2005). Experiments investigating innate avoidance behaviour have often measured the number of prey
attacked or the predator bias against one prey type. However, Jetz et al. (2001) noted that measures of this kind may miss the more subtle forms of innate avoidance behaviour, and suggested that the more sensitive measure of latencies may be preferable for work in this area.

In many past experiments individuals whose reactions varied too widely from their conspecifics were often excluded from experimental research (Speed 2000), whereas in fact this variability was indicative of an important ecological process. Marples and Kelly (1999) and Kelly and Marples (2004) provide evidence that unlearned wariness can be divided up into at least two distinct processes: neophobia, a refusal to make initial contact with the novel prey, to approach or peck at food, and dietary conservatism, a refusal to incorporate the novel prey into the diet. Predators can overcome neophobia of a food towards which they still exhibit dietary conservatism (Marples and Kelly 1999), thus supporting the theory that these are two distinct processes in innate avoidance behaviour. In order for this extended wariness to be displayed towards novel food, an alternative familiar food needs to be available (Rothschild 1984; Thomas et al. 2003; Kelly and Marples 2004), or else the bird may eat the novel food as it has no choice (Marples and Kelly 1999).

Neophobia may be deactivated within a matter of two to three minutes in domestic chicks (Marples and Kelly 1999; Marples et al. 2007). In captive wild species neophobia has been noted to last between 45 minutes for quail (Coturnix coturnix japonicas) (Marples and Brakefield 1995) to 2 hours for zebra finches (Taeniopygia guttata) (Kelly 2001). A profitable experience with one novel colour can turn off neophobia towards other novel-coloured prey (Schlenoff 1984; Marples et al. 2007) therefore, neophobia is unlikely to be an important line of defence against wild predators. Dietary conservatism, however, may cause wild birds to avoid a prey item for months on end, despite occasional sampling (Marples et al. 1998; Kelly 2001).
Marples and Kelly (1999) proposed that avoidance of a food type for such extended periods of time could hardly be attributed to a fear of "novelty".

Kelly and Marples (2004) noted that the addition of a novel odour to a novel colour cue significantly increased dietary conservatism, whereas they observed no effect on neophobia. This difference in effect of a multimodal signal further suggests that the two processes are distinct from one another. This result also adds weight to Rowe's (1999) argument that multimodal displays need to be studied holistically in order to fully appreciate their effects on predatory decisions.

Dietary conservatism may reduce predation pressure on a newly emerging aposematic species in such a way that the aposematic mutant spreads through the population. It may also allow conspicuousness to evolve before toxicity in the evolution of an aposematic species (Marples and Kelly 1999; Thomas et al. 2003, 2004; Marples et al. 2005). It seems imperative to differentiate between the two processes of innate avoidance if a complete understanding of the function of warning displays is to be achieved.

1.4.2 – Learning

Avoidance learning about aposematic prey species is a case of classic Pavlovian conditioning (Roper and Redston 1987; Ruxton et al. 2004). The predator learns to associate the conspicuous warning display (the conditioned stimulus) with the unprofitable effect (the unconditioned stimulus) to form a conditioned response, such as avoidance of the unpalatable prey (Pearce 1997). Therefore, when the conditioned stimulus is met in the future, the unconditioned stimulus will be recalled and subsequently the prey will be avoided (Mackintosh 1974; Speed 2000). The efficacy of the conditioned response is affected by the saliency of the conditioned and
unconditioned stimuli (Rescorla and Wagner 1972, Speed 2000). Conditioned stimuli that are visually conspicuous (Gittleman and Harvey 1980; Roper and Redston 1987; Roper 1994), distinctive (Gagliardo and Guilford 1993; Roper and Marples 1997a), novel (Shettleworth 1972; Turner 1984; Roper 1993) or waringly coloured such as red (Sillen-Tullberg 1985a, b; Guilford and Dawkins 1991), yellow, orange or white (Guilford and Dawkins 1991) have been shown to enhance avoidance learning. Both predator and prey species benefit from the predator acquiring a learned avoidance quickly, as the predator reduces the chances of poisoning, and fewer prey individuals need to be sacrificed in order for learning to occur (Moore et al. 1990; Marples and Roper 2004). There is, therefore, a selective advantage to a warning signal that speeds up the acquisition of a learned avoidance (Guilford and Dawkins 1991).

Rowe (1999) suggests that receivers may learn about multimodal displays faster than monomodal displays. When a receiver learns to avoid a multimodal signal, the component signals of the display may interact with one another in a variety of ways (Pearce 1997). One signal may potentiate, or aid, the association of the other signal with the unconditioned stimulus (Guilford and Dawkins 1991). Between-groups summation may also occur when the two signals presented together are learned faster than either of the component cues presented alone (Rowe 1999). This may occur as the multimodal signal presents a more salient display than either of the component cues, thus enhancing learning. The Rescorla and Wagner model (1972) predicts a similar effect; the model suggests that the speed of learning will be determined by the strength of the conditioned stimulus. A strong conditioned stimulus will increase the speed of learning, while a weak conditioned stimulus will slow learning down. Rothschild et al. (1984) suggested that accessory signals such as odour and sound could enhance the rate at which a learned avoidance may be acquired. Rowe (2002) reported an increase in learning in the presence of an artificial beeping sound. On the other hand,
overshadowing may also occur; one signal may become associated with the unconditioned stimulus, thus preventing an association between the other signal and the unconditioned stimulus (Pearce 1997). Roper and Marples (1997a) observed that almond odour overshadowed learning about a colour cue. This tends to happen when one of the conditioned stimuli is much more intense than the other (Rowe 1999). The effect of accessory signals on learning is discussed in more detail in Sections 1.2.2 and 1.2.3. However, there have been few demonstrations of a warning sound or smell enhancing avoidance learning of a warning colour, with the exception of Barnea et al. (2004), mentioned in Section 1.3.2.

Warning displays may increase the rate at which a learned avoidance is acquired through one of two mechanisms (Turner 1984; Guilford 1990). The distinctiveness of the display may increase the rate at which the prey are attacked, and therefore a learned avoidance is acquired faster, as observed by Gittleman and Harvey (1980). Alternatively, there may be something inherent about the warning signal that speeds up learning without increasing attack rate (Roper and Redston 1987).

1.4.3 – Memory

Predators need to remember their learned avoidance if defended prey are to be avoided on future encounters (Guilford and Dawkins 1991), remembering a learned avoidance is of benefit to the predator as once educated it avoids the defended prey. Prey species also benefit from having memorable signals, as they do not need continually to re-educate their predators (Moore et al. 1990; Guilford and Dawkins 1991). While much is known about how warning displays increase learning rates, relatively little is known about how signal design affects memorability, that is, the resistance of long-term memories to forgetting (Speed 2000). Stimuli that have been
noted for their ability to enhance learning rates may also improve memorability (Roper 1994; Speed 2000); therefore much of the work that relates to the former may also be applicable to the latter. Despite the limited research conducted on the memorability of warning signals, it is known that certain distinctive visual cues such as conspicuousness (Gittleman and Harvey 1980; Roper and Redston 1987; Guilford and Dawkins 1991), novelty (Shettleworth 1972; Roper 1990) and warning colours (Sillen-Tullberg 1985b) are learned and remembered better than other visual cues. Yachi and Higashi (1998) went so far as to suggest that increased memory retention of conspicuous signals may help to explain the evolution of aposematic displays.

As learning rates are frequently negatively correlated with forgetting rates, it is often difficult to separate out the effects of the two psychological processes (Speed 2000). If recall is better for one signal than another, it may be that the first signal was learned to a greater extent, or that it is less prone to forgetting. In order to differentiate between the processes of greater learning and better memory, Shettleworth (1998) suggests the use of multiple tests of memory. One test soon after learning can be used to establish whether learning occurred to the same extent with various signals, and another some time later to determine how well these signals are remembered over time. Two warning signals may elicit the same learning rate, but one may be more memorable than the other and therefore confer more protection to the prey species displaying it (Speed 2000).

As the intensities of the conditioned and unconditioned stimuli increase, the association between them also increases, resulting in a stronger conditioned response to the conditioned stimulus (Rescorla and Wagner 1972; Pearce 1997; Section 1.4.2). The intensity of the conditioned and unconditioned stimuli may have similar effects on memorability (Mackintosh 1974). Anything that increases a signal’s conspicuousness may enhance its memorability (Roper and Redston 1987). Accessory signals such as
warning odours may therefore enhance memorability of the warning display (Guilford and Dawkins 1991). Kehoe et al. (1994) demonstrated in the rabbit, prolonged memory of a multimodal in comparison to a monomodal signal. There has been limited research investigating how olfactory and auditory cues affect memorability and interact with visual signals as part of a multimodal display (Speed 2000). The work conducted thus far has been reviewed in Sections 1.2.2 and 1.2.3 of this chapter.

1.4.4 – Generalisation

Generalisation is a feature of learning (Rowe 1999). If two signals are similar to one another, the conditioned response learned towards one may be generalised towards the other (Pearce 1997). Ham et al. (2006) noted that cues which signal a negative experience may be more readily generalised to other cues than those that denote a positive experience.

If signals are relatively similar, a receiver may save time by generalising between them and applying the same response to both, rather than engaging in a time-consuming discrimination task (Chittka and Osorio 2007). Animals generalise less between increasingly dissimilar signals (Chittka and Osorio 2007) and so the presence of an additional signal may reduce generalisation (London 1954). There are several examples of reduced generalisation between multimodal signals in comparison to monomodal signals (Fink and Patton 1952; Heineman and Chase 1970). Guilford and Dawkins (1991) noted that attributes which enhance discrimination of the signal may be important for signal design. Rowe (1999) suggests that the addition of an olfactory or auditory cue may reduce generalisation and therefore enhance discrimination learning; however, this has not as yet received any empirical investigation.
1.4 - MIMICRY

Many undefended insect species (mimics) gain protection by mimicking the warning signals of aposematic species (models), thereby fooling the predator into generalising the learned avoidance of the model to the mimic species (Bates 1862; Duncan and Sheppard 1965). Mimics and models are not always sympatric and may be separated spatially (Shettleworth 1972; Waldbauer 1988a), temporally (Shettleworth 1972; Waldbauer 1988b), or both (Joron 2003). As a result, long-term memorability of learned avoidance of the model is important for the protection of the mimic (Guilford and Dawkins 1991).

Mimicry erodes the unpalatable model’s protection from predation, and it is therefore to the model’s benefit to evolve signals that make it distinguishable from its mimics (Rowe 1999). Therefore, if the model’s visual warning signal has been mimicked, the addition of an olfactory or auditory warning display may restore the model’s protection, as it could enhance discrimination between the model and mimic (Moore et al. 1990; Guilford and Dawkins 1991). In order for the predator to generalise its learned avoidance from the multimodal model species, the mimic may have to replicate both component signals of the model’s warning display (see Section 1.4.4). This creates an arms race between the model and mimetic species, as the model evolves away from the mimic, and the mimic evolves towards the model (Rowe 1999).

Despite the fact that there are many examples of multimodal mimicry by insect species (Rothschild 1984; Moore et al. 1990), there has been no empirical investigation of whether mimics of multimodal models need to mimic all components of the multimodal signal, or whether mimicry of the visual component alone is sufficient to give full protection. There is evidence to suggest that even poor visual mimics of the model can often gain some level of protection (Cuthill and Bennett 1993; Dittrich et al. 1993), though they are not as well protected as accurate mimics (Azmeh et al. 1998).
1.6 – A MODEL SYSTEM TO INVESTIGATE AVIAN PREDATORY DECISIONS

1.6.1 – The domestic chick as a model avian predator

In an effort to understand predator psychology and responses to defended prey, much research has been conducted using the domestic chick (*Gallus gallus domesticus*) as a model avian predator (Härlin and Härlin 2003).

Kelly and Marples (2004) reported that conclusions about dietary conservatism and neophobia inferred from experimentation on chicks were also applicable to zebra finches, which they used as a model passerine. Despite similarities in the behaviour of domestic chicks and zebra finches, chicks were much less conservative in their food choice (Kelly and Marples 2004). Lindström *et al.* (1999) noted that naïve hand-reared great tits (*Parus major*) showed less innate avoidance than wild caught adult birds. This suggests that innate avoidance behaviour observed using chicks in the laboratory may underestimate the avoidance behaviour of wild adult birds, a view which is supported by Rowe (1999).

For several reasons, one must proceed with caution when generalising results observed using domestic chicks in the laboratory to how wild birds respond to live insect prey (Marples *et al.* 1998). Firstly, domestic chicks have been bred to be non-selective in their diet, thereby maximising weight gain (Mench 2002). This may make chicks less selective in their food choices than other bird species (Kelly and Marples 2004). Secondly, chicks used in laboratory experiments often come from the same hatchery and hatching batch. Due to this, and because the chicks have been bred as a domestic species, they may have low genetic variability. Wild birds tend to have greater genetic variability than domestic chicks, which may cause greater variability in their predatory decisions (Marples and Brakefield 1995). Thirdly, a predator’s willingness to sample novel food may be affected by physiological condition and
hunger level. During experimentation, chicks tend to be in good condition and are food deprived for a set time period to standardise their hunger levels; however, it is much more difficult to standardize wild birds' body condition or hunger levels (Marples et al. 1998). Finally, newly hatched domestic chicks in laboratory experiments are naïve, whereas wild adult birds have extensive foraging experience which may alter their predatory decisions (Kelly and Marples 2004). Biases may also be passed on culturally between parents and offspring (Doherty and Cowie 1994; Lindström 1999), which may mean that precocial species such as domestic chickens may exhibit less avoidance behaviour than altricial species.

Despite the disparity between the results for domesticated and wild birds, it appears that chicks are a good starting point for elucidating the mechanisms of warning displays, and many successful studies have been conducted using chicks as model predators (Shettleworth 1972; Sillen-Tullberg 1985a; Marples and Roper 1997; Rowe and Guilford 1999a; Jetz et al. 2001; Lindström et al. 2000; Skelhorn and Rowe 2005). However, it is clear from the above discussion that assumptions about avian food preferences made from results obtained using domesticated chicks need to be verified using wild birds. Given the time constraints of this research project and the paucity of knowledge about the effects of non-visual signals on innate and learned predatory behaviour, most of the research was conducted using chicks in the lab. However, this project also aimed to replicate experiments of particular interest in the wild to determine how well the findings reflect the behaviour of avian predators in general.

1.6.2 - Artificial prey as part of the model system

Artificial prey such as pastry baits, plastic pins and dyed chick starter crumbs have also been used in a great many experiments (Sillen-Tullberg 1985a; Roper and Redston 1987; Rowe and Guilford 1996; Skelhorn and Rowe 2005, 2006; Marples et
al. 2007). This allows all components of the warning signal to be controlled for, modified and examined individually. However, in using artificial prey, many signals that are present in live insect prey may be absent (Lindström 1999). This may alter the predators' responses to the prey items due to a lack of movement and other signals present in live prey. While model systems are a good starting point for the investigation of predator responses to aposematic displays, it is essential that any results achieved in a laboratory situation be verified in a more natural situation using adult, wild birds, and live insect prey.

1.7 - OUTLINE OF THE RESEARCH

The research conducted for this project sought to investigate further the effect of olfactory and auditory warning signals on innate and learned avoidance behaviour and the memory of the learned avoidance. The two distinct processes of innate avoidance behaviour, neophobia and dietary conservatism, are examined in Chapters 3 and 4. In particular, Chapter 3 investigates the effects of pyrazine odour on neophobia and dietary conservatism towards yellow and red crumbs. It also examines the effect which familiarity with one of the component cues in the multimodal signal has on avoidance behaviour, in an attempt to address further whether component signals of a multimodal display need to be novel in order for a behavioural response to be observed, as touched on by Rowe and Guilford (1999a) and Marples and Roper (1996). Chapter 4 examines the effect of a warning sound, buzzing, on neophobia and dietary conservatism of novel yellow and red prey. This chapter also provides the first empirical investigation into the effects of a trimodal warning signal on neophobia and dietary conservatism. Chapters 3 and 4 also compares the different methods of
measuring innate avoidance behaviour, namely latency measures such as neophobia and dietary conservatism, count measures such as number of crumbs attacked, and a derived measure, such as attack bias, as used by Rowe and Guilford (1996, 1999a, b).

The remainder of the thesis concentrates on the effects of multimodal warning displays on learned avoidance and the memory of this avoidance. Chapter 5 examines whether pyrazine odour enhances learned avoidance of unpalatable yellow or red crumbs, and how well the learned avoidance was remembered after a retention interval. It also compares the avoidance of accurate and inaccurate mimics of the unpalatable model crumbs, in order to examine how interactions between the component cues of the multimodal display affect memory. Finally, it investigates whether the presence of pyrazine odour affects generalisation of the learned response towards the defended prey species to palatable species in close proximity. Such generalisation based on a secondary cue has not been considered in previous research.

Wild robins were used as experimental subjects in part of a field study, reported here as Experiment 8 in Chapter 6, designed to investigate whether pyrazine odour enhanced learned avoidance of unpalatable yellow baits in wild birds. Finally, the effect of a warning sound, buzzing, on learned avoidance and memory of unpalatable yellow crumbs is examined in Experiment 9 of Chapter 7. The results of these experiments, taken together, shed more light on how olfactory and auditory signals operate to alter avian predator avoidance behaviour, in particular adding knowledge to the differentiation between neophobia and dietary conservatism, and to how multimodal displays affect memory.
CHAPTER 2 – MATERIALS AND METHODS
Day-old male domestic chicks (Ross strain) were used as model avian predators for the laboratory experiments. They were delivered to the laboratory on the day of hatching from a commercial hatchery (Carlton Hatchery, Monaghan, Ireland), and housed under license number B100/3802 held by E. Siddall. The chicks were housed in a wooden pen (150 cm long x 60 cm wide x 60 cm deep), the floor of which was covered in wood shavings. They were subject to a 12 L: 12 D light cycle using uncovered fluorescent lights, augmented by natural light during part of the 12 L phase. The temperature of the room was maintained at 24 – 25 °C using radiators and ceramic heat lamps. Water was provided *ad libitum* throughout the experiment, and chick starter crumbs were provided *ad libitum* except for one hour prior to testing, when chicks were food deprived. All food deprivation was carried out in accordance with EU guidelines (86/609/EC). The colour of the crumbs provided in the home box depended on the particular experiment, discussed below.

On the day of arrival, all chicks were individually marked on their heads using non-toxic permanent marker pens. This procedure appeared to have no adverse effects on the study subjects and they did not appear to respond to the marks on their own or other chicks’ heads.

The laboratory consisted of two rooms separated by a door. All chicks were housed in the first room. Chicks in treatments that were given an odour or sound cue were tested in the second room. An extractor fan ran in both rooms for the duration of each experiment, drawing air to the outside of the building. The door between the two rooms was kept shut at all times when odour or sound were present in the second room.
When each experiment was completed the chicks were humanely euthanized, either by gassing with CO₂ or by euthathol injection into the intraperitoneal cavity.

Experiment 8 used wild robins as the experimental subjects; the methods for this experiment are discussed in Section 6.2 of Chapter 6.

2.2 – ARTIFICIAL PREY

2.2.1 - Colour cues

The prey used were chick starter crumbs (Connolly’s Red Mills, 20% broiler chick starter crumb), which were coloured using food dye. The crumbs were dyed either green or one of two colours thought to be classically aposematic, yellow and red (Schuler and Roper 1992). Prior to being dyed, the crumbs were sieved in order to remove dust that would otherwise have interfered with the dying process. This also helped to standardise the crumb size. In Experiment 1 bright green crumbs were dyed using O’Brien’s™ (Citywest, Dublin 24, Ireland) Green Mint KBT. Ten ml of the dye was diluted to make 40 ml of solution using distilled water, and mixed with 100 g of chick starter crumbs. For all subsequent experiments pale green crumbs were dyed using Sugarflair Colours Ltd.™ (Benfleet, Essex, UK) Spruce Green. Half a millilitre of the dye was diluted to make 90 ml of solution using distilled water, which was then mixed with 150 g of chick starter crumbs. This is the same concentration used by Skelhorn and Rowe (2005, 2006).

Two different yellow dyes were used depending on the experiment in question. For the neophobia and dietary conservatism experiments, O’Brien’s™ (Citywest, Dublin 24, Ireland) Lemon Yellow T dye was used; 10 ml of dye was diluted to make a solution of 40 ml with distilled water, which was then mixed with 100 g of sieved
This yellow concentration was also used in the first learning experiment, Experiment 5. For each subsequent learning experiment the yellow crumbs were dyed using Sugarflair Colours Ltd.™ (Benfleet, Essex, UK) Egg Yellow; 0.5 ml of the dye was diluted to make 90 ml of solution using distilled water, and then mixed with 150 g of chick starter crumbs.

Supercook™ red food dye (Sherburn-in-Elmet, Leeds, UK) was used to make the red crumbs in all the experiments; 2 ml of the dye was diluted with distilled water to make 90 ml of solution, which was then mixed with 150 g of sieved starter crumbs. This is the same dye concentration used by Skelhorn and Rowe (2005, 2006).

After being dyed, the crumbs were spread out on sheets of paper and allowed to dry for 24 hours prior to use in experiments. The crumbs were disturbed periodically during the drying process in order to prevent them sticking together. Each of the colours produced comparable saturations to the researcher's eye.

2.2.2 - Unpalatable cue

In the learning trials the aposematically coloured crumbs were unpalatable. The crumbs were made unpalatable prior to colouring using 2.5% W/V denatonium benzoate (MacFarlan Smith Ltd.™), commercially available as “bitrex”. This is a bitter substance that has been used in previous studies to induce taste aversions in chicks (Skelhorn and Rowe 2005). In Experiment 5 the yellow crumbs were made unpalatable using 30 ml of 2.5% bitrex diluted with 30 ml distilled water and mixed with 100g of chick starter crumbs; however, as discussed in Section 5.2.4, this concentration appeared to be too strong. Therefore, in all subsequent learning experiments a weaker concentration of bitrex was used (five drops of 2.5% bitrex, approximately 0.2 ml, were added to 90 ml of tap water, which was then mixed with
150g of sieved chick starter crumbs). The crumbs were then allowed to dry for 24 hours prior to colouring.

2.2.3 - Odour cues

In the treatments that were exposed to the pyrazine odour, one drop (approximately 0.04 ml) of pyrazine solution was placed beneath the aposematically coloured crumbs in the experimental arena (Fig. 2.1). The pyrazine solution consisted of 100 μl of 2-isobutyl-3-methoxypyrazine diluted to 1000 ml using distilled water, as used by Marples and Roper (1996) and Rowe and Guilford (1996, 1999a).

The pyrazine solution was stored in a sealed container in the odour room, in order to prevent cross-contamination of the odour to the non-odour room. A fresh solution of pyrazine was made every four months. Pyrazine is an extremely pervasive odour, and the undiluted solution was kept in a fume cupboard in a different laboratory from the one where the chicks were held.

2.2.4 - Sound cues

The buzzing sound was a recording of an agitated bumble bee (*B. terrestris*) trapped in a net, recorded using a Sony Minidisc (M2 N505 type-R) player, which was similar to the sound used by Rowe and Guilford (1999b). It was played back using the same minidisc player through four Cambridge Soundworks speakers placed on each side of the test arena (Fig. 2.1). The buzzing sound had a maximum volume of between 87 - 95 dB between frequencies of 1 kHz and 5 kHz, measured using the software package Raven™ 1.2.1., Build 27.3 Update 22.3. The buzzing sound was played at between 65 – 70 dB (measured using a Roline R0-1350 sound level meter) when a chick approached a well that contained an aposematically coloured crumb, and the sound was stopped when the chick left the well, dropped or swallowed the crumb.
The experimental arena was a 30 cm long x 21 cm wide x 22 cm deep cardboard box, with a 10 cm long x 21 cm wide section divided off using chicken wire (Fig. 2.1). Two “buddy chicks”, previously fed to satiation, were placed in this smaller section, and the test chick was placed in the larger section. Buddy chicks reduce the stress of isolated test chicks (Marples and Roper 1996; Skelhorn and Rowe 2005). No buddy chicks were used as experimental chicks.

A Perspex™ feeding tray, 20 cm in diameter, was used to present the artificial prey in the test arena. The tray consisted of two layers of Perspex™ (Fig. 2.1), each of which was punctured by 24 wells, 12 mm in diameter. The wells in the top layer had a mesh floor, and the wells in the bottom layer had a solid floor. Twelve green and twelve aposematically coloured crumbs were presented, one in each of the 24 wells on top of the mesh. The spatial arrangement in which the crumbs were presented was determined using a randomly generated map, created by drawing numbers from a hat. The map was constrained so that no more than four of the same crumb colour could occur in adjacent wells. During an individual trial a different map was used for each chick. There were 32 random maps, and the maps were rotated a quarter turn to the left each time they were used, to ensure that no chick met an identical map twice.

During the odour experiments, each well in the bottom layer of the feeding tray below the mesh floor contained a small piece of filter paper (1 cm²). Immediately prior to the start of each trial of an odour treatment, a drop of the pyrazine solution was placed on the filter paper beneath the wells containing aposematically coloured crumbs, and a drop of water was placed beneath the wells containing green crumbs. In the odour treatments, pyrazine therefore may have acted as an additional discriminatory
only the aposematically coloured and not the green crumbs. In the non-odour
treatments, a drop of water was placed in all the wells.

During the sound experiments nothing was placed in the bottom layer of the
tray, but the buzzing sound was played each time the chick approached a well
containing an aposematically coloured crumb.
Figure 2.1 The test arena, showing the feeding tray, the chicks, and indicating the placement of speakers around the experimental arena during the sound experiments.
2.4 – PRE-TRAINING

On the day of their arrival (day one), the chicks were pre-trained in pairs to accustom them to the test arena. They were allowed to eat chick starter crumbs the same colour as their home food from the feeding tray (in the absence of any odour or sound) for two 10-minute sessions. Each chick then received between four and six more pre-training sessions of five minutes each, accompanied by two buddy chicks in the buddy chamber. By the end of day one, all of the chicks ate readily from the feeding trays.

2.5 – INNATE AVOIDANCE EXPERIMENTS

The chicks were given green food in their home box for the entirety of the experiment so that green was familiar to them, and they were pre-trained on green food as described in Section 2.4. For chicks that needed to be familiar with a colour or odour cue prior to testing, they were given a forty minute social training trial with the familiar food. This social training was carried out in the presence of all the other chicks from their treatment. The remainder of the training trials consisted of four one-minute training sessions, during which there was one crumb with which they were to become familiar in each well of the feeding tray. By the end of these training sessions each chick readily ate the familiar crumb type.

Once training was completed on day two, the chicks were food deprived for one hour prior to testing and were then offered a three-minute choice test between twelve green crumbs and twelve aposematically coloured crumbs, in which all the crumbs were palatable. During these trials the time taken to peck one crumb of each colour
was noted as a measure of neophobia. Once neophobia was overcome, the time taken to eat three crumbs of each colour in one trial was noted as a measure of dietary conservatism. If a chick ate three aposematically coloured crumbs in one trial, it was deemed to have incorporated that food type into its diet and therefore to have overcome dietary conservatism. This is the same measure used by Marples et al. (2007) who observed that once a chick ate three crumbs of one colour they continued to eat the crumbs; therefore, they deemed the eating of three crumbs to be a satisfactory criterion for the deactivation of dietary conservatism. If the chick did not eat three of each colour of crumb in the first session it was re-tested an hour later. A maximum of six such tests were conducted for each chick, one on day two, three on day three and two on day four. Thus, each chick had up to 18 minutes' cumulative exposure time to overcome dietary conservatism. This 18-minute time limit was calculated from the point the chick overcame neophobia, therefore standardizing the time available to overcome dietary conservatism across all chicks, regardless of how long they took to overcome neophobia.

The number of crumbs of each colour eaten by each chick was also noted, which allowed the ingestion of each crumb colour to be compared. A measure analogous to Rowe and Guilford's (1996, 1999a, b) "attack bias" was calculated from these data. This allowed more direct comparisons of the results from this thesis to those of other researchers who used the attack bias measure. However, in this thesis it was decided to call this measure "eating bias" as opposed to "attack bias" as Rowe and Guilford (1996, 1999a, b) termed it, since the measure reported the proportion of crumbs of each colour eaten rather than crumbs which were merely attacked.

The number of crumbs of each colour that were eaten, and the eating bias measures were only calculated for the first three-minute trial. All chicks were allowed to complete the full three minutes of the first trial regardless of whether they overcame
dietary conservatism. This allowed them to eat as many of the twelve crumbs of each colour as they wished, rather than finishing the trial after they had attacked three aposematically coloured crumbs, which would have prevented the use of the number of crumbs eaten as a measure. Chicks who overcame their dietary conservatism during the first trial were not tested in subsequent trials; therefore, to allow comparison of the behaviour of all chicks, these measures could only be calculated for the first trial.

2.6 – LEARNING EXPERIMENTS

The chicks were given brown food at home for the entirety of the experiment, and they were pre-trained on brown food as described in Section 2.4. Therefore at the start of testing both crumb colours were novel to the chicks.

2.6.1 - Learning trials

Once pre-training was complete, testing began. On day two, the chicks were deprived of food for approximately one hour before their first learning trial, during which they were offered 12 unpalatable aposematically coloured crumbs and 12 palatable green crumbs placed in a random order in the feeding tray.

The number of aposematically coloured and green crumbs attacked during the learning trial was noted. An attack was defined as when the chick either picked up and dropped or picked up and swallowed the crumb. However, if the chick merely nudged the crumb with its beak this was not defined as an attack. It was assumed that as the chick did not taste the crumb in this instance, it gained no learning experience from this behaviour (Speed 2000). The trial continued either until the chick had attacked 12 of
the 24 crumbs, or for three minutes, whichever occurred first. A total of seven learning trials were conducted, three on each of days two and three, and one on day four. However, in Experiment 5 only six learning trials were conducted. Once the learning trials were complete each treatment group was subdivided into two extinction groups, one to be tested three hours after learning was completed and the other to be tested 96 hours after learning was completed.

This thesis used two methods to measure avoidance learning. Firstly, as reported by Skelhorn and Rowe (2006), the total number of unpalatable crumbs attacked by each chick during the learning trials was calculated as a measure of learning. This measure allowed differences in overall attack levels to be compared, even when there were differences in attack rate during the first learning trial. Secondly, the number of unpalatable crumbs attacked during each trial was analysed, as this allows differences in learning rates to be examined more closely.

2.6.2 - Extinction Trials

Three hours after completion of the final learning trial, chicks in the 3-hour retention interval group took part in an extinction trial in which all crumbs were palatable. This trial investigated whether learning had occurred, as it tested for the presence of the conditioned response (the avoidance of the unpalatable crumbs) towards the conditioned stimulus (the warning display) in the absence of the unconditioned stimulus (the bitrex) (Pearce 1997). Memory formation processes in the chick may continue for several hours after learning (Tiunova et al. 1998; Hale and Crowe 2002). Therefore, during this three hour period after learning, a process called “consolidation” may occur, during which recent memories are committed to long term memory, and learned performance may continue to improve. This first extinction trial was therefore considered a more accurate measure of the chicks’ final learned
avoidance level than the final learning trial. This 3-hour trial also allowed the processes of learning and memory to be separated out somewhat as suggested by Shettleworth (1998) and discussed in Section 1.4.3.

On day seven, chicks in the 96-hour retention interval group received an extinction trial, designed to test the chicks' memory of the learned avoidance. The numbers of green and aposematically coloured crumbs attacked during the consolidation and extinction trials were noted.

2.7 - DATA ANALYSIS

From investigation of histograms and analysis using the Kolmogorov-Smirnov test, it was determined that the data were not normally distributed (Dytham 2003; Zar 2005). As the data did not conform to the assumptions of parametric statistics and could not be transformed by any standard method, the data were analysed using non-parametric statistics, Mann-Whitney U and Kruskal-Wallis tests (Dytham 2003; Zar 2005). When Kruskal Wallis tests gave significant results across treatment groups, Dunn's post-hoc test was used to make pairwise comparisons (Zar 2005).
CHAPTER 3

The effect of pyrazine odour on innate avoidance behaviour towards aposeatically coloured food

Acknowledgement and authorship

Both experiments reported in this chapter were designed and analysed by E. Siddall. Dr. Marples and Dr. Thomas contributed to the design of the experiments. The data for Experiment 1 of this chapter were collected by E. Siddall. The data for Experiment 2 was collected by J. Beggs during her undergraduate moderatorship project which was co-supervised by Dr. Brown and E. Siddall.
3.1 - EXPERIMENT 1 - The effect of pyrazine odour on neophobia and dietary conservatism towards yellow crumbs

3.1.1 - INTRODUCTION

Previous work has shown that pyrazine odour, which is commonly used as part of insect warning displays (Rothschild 1961; Moore et al. 1990; Marples and Roper 2004), may enhance innate avoidance behaviour exhibited by domestic chicks towards novel and familiar yellow prey (Rowe and Guilford 1999a, b). This effect is no longer detected if the pyrazine odour is familiar to the chicks (Rowe and Guilford 1999b). These previous studies did not differentiate between the two processes of innate avoidance, neophobia and dietary conservatism (Marples and Kelly 1999). The experiment reported below sought to investigate whether pyrazine odour affected neophobia and dietary conservatism in different ways. It further investigated whether pyrazine could enhance innate avoidance of novel yellow prey even when the odour was familiar.

Rowe and Guilford (1999a, b) familiarised the chicks with the pyrazine odour in the presence of palatable brown chick starter crumbs. This may have caused the chicks to form a positive association between the pyrazine odour and the palatable food. However, given that pyrazine is a commonly used warning signal but is rare in other contexts, (Rothschild 1961; Guilford et al. 1987; Moore et al. 1990; Rowe and Guilford 1996, 1999a) if a predator has previously encountered pyrazine in the wild, it is likely that it will have done so in a negative context, thus creating an association between pyrazine and unprofitability. Therefore, the pairing of pyrazine with a positive reinforcer in the Rowe and Guilford (1999a) study was rather unnatural. If the pyrazine odour was associated with a negative experience during the chicks' initial training, the
results observed may have been very different. In order to control for this effect an additional treatment was included in this current experiment; chicks were familiarised with pyrazine odour in the presence of unpalatable brown crumbs.

3.1.2 – METHODS

The experiment was conducted over two weeks with Treatments 1 and 3 replicated in each week. Mann-Whitney U tests were conducted on the data from the two weeks and no significant differences were found; therefore, the data were combined for analysis. This gave a total of 28 replicates in treatments one and three, and 14 replicates in every other treatment.

This experiment differed from the other innate avoidance experiments discussed in this thesis (Experiments 2, 3 and 4) as the chicks were given brown as opposed to green food at home and were also pre-trained on day one using brown food. The pre-training method is described in Section 2.4. On day two the chicks were given a forty-minute social training session and four one-minute individual training sessions on each familiar food type (Table 3.1). Once training was completed on day two, the chicks were offered 12 green crumbs and 12 yellow crumbs in the presence or absence of pyrazine depending on the treatment (Table 3.1), and measurements of innate avoidance towards the novel signals were recorded as discussed in Section 2.5.
Table 3.1: Treatments received during the training and test trials, indicated as crumb colours and odours. All crumbs were palatable.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Training food</th>
<th>Test food</th>
<th>Test for response to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green; brown</td>
<td>Green; yellow</td>
<td>Novel yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green; yellow</td>
<td>Green; yellow</td>
<td>Familiar yellow</td>
</tr>
<tr>
<td>3</td>
<td>Green; brown</td>
<td>Green; yellow plus pyrazine</td>
<td>Novel yellow and novel pyrazine</td>
</tr>
<tr>
<td>4</td>
<td>Green; yellow</td>
<td>Green; yellow plus pyrazine</td>
<td>Familiar yellow and novel pyrazine</td>
</tr>
<tr>
<td>5</td>
<td>Green; palatable brown plus pyrazine</td>
<td>Green; yellow plus pyrazine</td>
<td>Novel yellow and familiar pyrazine associated with palatable crumbs</td>
</tr>
<tr>
<td>6</td>
<td>Green; unpalatable brown plus pyrazine</td>
<td>Green; yellow plus pyrazine</td>
<td>Novel yellow and familiar pyrazine associated with unpalatable crumbs</td>
</tr>
</tbody>
</table>

3.1.3 – RESULTS

There was a significant difference in the time taken to overcome neophobia towards yellow crumbs across the groups (Kruskal-Wallis test, $\chi^2 = 37.341$, d.f. = 5, $p < 0.001$, Fig. 3.1.1). Yellow alone did not elicit a neophobic response, as there was no significant difference between the time to peck novel and familiar yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 2 NS). Novel pyrazine odour prolonged neophobia of yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 3 $p < 0.001$) but only when the colour was novel to the chicks (Dunn’s post-hoc test Treatment 2 vs. 4 NS). The familiar pyrazine odour also prolonged neophobia of novel yellow food (Dunn’s post-hoc test Treatment 1 vs. 5 $p < 0.01$ and Treatment 1 vs. 6 $p < 0.01$). The method of odour familiarisation, either with palatable or unpalatable crumbs, had no effect (Dunn’s post-hoc test Treatment 5 vs. 6 NS).

In addition to the responses towards the yellow crumbs, the chicks’ reactions to the familiar green crumbs under the test conditions were enlightening. There were significant differences between the treatments in the time taken to peck the first
experimental green crumb (Kruskal-Wallis test, $\chi^2 = 13.484$, d.f. = 5, $p < 0.05$, Fig. 3.1.1). Chicks familiar with green and yellow crumbs took less time to peck their first green crumb during testing than chicks familiar with only green (Dunn’s post-hoc test Treatment 1 vs. 2 $p < 0.05$). The chicks took longer to peck the green crumbs in the presence of familiar yellow crumbs that smelt of pyrazine than in the presence of odourless familiar yellow crumbs (Dunn’s post-hoc test Treatment 2 vs. 4 $p < 0.05$), which further suggests that the presence of the pyrazine odour enhanced avoidance of the green crumbs despite the chicks being familiar with them.

Somewhat surprisingly, neophobia of novel yellow crumbs was overcome significantly faster than neophobia of familiar green crumbs in Treatment 1 (Mann-Whitney U test, $U = 205.00$, $n = 28, 28$, $p < 0.01$, Fig. 3.1.1). Neophobia of familiar yellow crumbs that smelt of pyrazine was also overcome significantly faster than neophobia of familiar green crumbs in Treatment 4 (Mann-Whitney U test, $U = 39.00$, $n = 14, 14$, $p < 0.01$). There were no significant differences in the time to overcome neophobia of yellow and green crumbs in the other treatments.
Figure 3.1.1 - The mean latency (±s.e) (in seconds) to overcome neophobia of green and yellow crumbs (indicated by the colour of the bars) in the presence and absence of pyrazine odour. Treatments 1 and 3 had 28 replicates each, while all other treatments had 14 replicates.

Please note the change in scale between the two graphs (Fig. 3.1.1 and Fig. 3.1.2), demonstrating that the latency to overcome neophobia was very short compared to the latency to overcome dietary conservatism.
There were significant differences in the times taken to overcome dietary conservatism of yellow crumbs across the six treatments (Kruskal-Wallis test, $\chi^2 = 37.341$, $d.f. = 5$, $p < 0.001$, Fig. 3.1.2). The chicks showed significantly higher levels of dietary conservatism towards novel than familiar yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 2 $p < 0.001$), which suggests that familiarisation with yellow deactivated dietary conservatism towards yellow crumbs. Dietary conservatism of novel yellow prey was prolonged when novel pyrazine odour was present (Dunn’s post-hoc test Treatment 1 vs. 3 $p < 0.01$). Novel pyrazine odour also appeared to
enhance dietary conservatism towards familiar yellow prey, although this only approached statistical significance (Dunn’s post-hoc test Treatment 2 vs. 4, critical value = 23.35, mean rank difference = 21.89). Dietary conservatism of novel yellow prey was prolonged to the same extent by pyrazine odour regardless of whether the odour was novel or familiar (Dunn’s post-hoc test Treatment 3 vs. 5 NS, Treatment 3 vs. 6 NS), and this result occurred irrespective of whether the chicks associated the pyrazine odour with palatable or unpalatable food (Dunn’s post-hoc test Treatment 5 vs. 6 NS).

There were no significant differences in the time taken to overcome dietary conservatism of green crumbs across the six treatment groups (Kruskal-Wallis test, \( \chi^2 = 9.094, d.f. = 5, \) NS, Fig. 3.1.2). However, when the times for green and yellow crumbs to be eaten were compared for each treatment some differences were found. There were no significant differences between the time taken to overcome dietary conservatism of familiar green and novel yellow crumbs in Treatment 1; however, when the novel yellow crumbs smelt of pyrazine in Treatment 3, the chicks took significantly longer to overcome dietary conservatism of the yellow than the green crumbs (Mann-Whitney U test, \( U = 164.00, n = 28, 28, p < 0.001 \)). This result was also observed when the pyrazine odour was familiar to the chicks (Mann-Whitney U test; Treatment 5, \( U = 11.00, n = 14, 14, p < 0.001 \); Treatment 6, \( U = 44.00, n = 14, 14, p < 0.05 \)). When the chicks were familiar with both the green and yellow crumbs (Treatments 2 and 4) they showed significantly more dietary conservatism towards the green crumbs when pyrazine was absent (Treatment 2, Mann-Whitney U test, \( U = 46.00, n = 14, 14, p < 0.05 \), but no difference in dietary conservatism towards the two colours when pyrazine was present (Treatment 4, Mann-Whitney U test, \( U = 75.50, n = 14, 14, \) NS).
When we consider the number of crumbs of each colour eaten during the first trial it is clear that there were significant differences in the number of yellow crumbs eaten across the treatments (Kruskal-Wallis test, $\chi^2 = 54.119$, d.f. = 5, $p < 0.001$, Fig 3.1.3). Chicks ate significantly more familiar than novel yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 2 $p < 0.01$, Fig. 3.1.3). When the novel yellow crumbs smelt of novel pyrazine, the chicks ate fewer crumbs again (Dunn’s post-hoc test Treatment 1 vs. 3 $p < 0.01$). This effect was also observed when the pyrazine odour was familiar to the chicks (Dunn’s post-hoc test Treatment 1 vs. 5 $p < 0.05$; Treatment 1 vs. 6 $p < 0.05$), and the method with which the chicks were made familiar with the pyrazine odour had no effect on this (Dunn’s post-hoc test Treatment 5 vs. 6 NS). The addition of novel pyrazine odour to familiar yellow crumbs had no effect on the number of crumbs the chicks were willing to eat during the first three-minute trial (Dunn’s post-hoc test Treatment 2 vs. 4 NS).

The chicks ate significantly different numbers of green crumbs across the treatment groups (Kruskal-Wallis test, $\chi^2 = 11.876$, d.f. = 5, $p < 0.05$, Fig. 3.1.4). When the novel pyrazine odour was present, the chicks ate significantly fewer green crumbs than in its absence (Dunn’s post-hoc test Treatment 1 vs. 3, $p < 0.05$). When the odour was familiar to the chicks, it did not decrease the number of green crumbs they ate (Dunn’s post-hoc test Treatment 1 vs. 5 NS, Treatment 1 vs. 6 NS). However when the yellow was familiar they ate the same number of green crumbs as when it was novel (Dunn’s post-hoc test Treatment 1 vs. 2 NS), suggesting that it is the novelty of the smell which causes these changes rather than the novelty of yellow.
Figure 3.1.3 – The mean number (±s.e) of familiar green and novel yellow crumbs eaten during the first trial in the presence and absence of the pyrazine odour.

3.1.3a - The effect of analysis method on results

Rowe and Guilford (1996, 1999a, b) used attack bias as a measure of innate avoidance behaviour. An analogous measure, eating bias, was included as a measure in this thesis for comparative purposes (see Section 2.5). There were significant differences between the eating bias across the six treatment groups (Kruskal-Wallis test, $\chi^2 = 27.443$, d.f. = 5, p < 0.001, Fig. 3.1.4). Positive values in Fig. 3.1.4 indicate a preference for green crumbs, and negative values a preference for yellow crumbs. When the yellow crumbs were novel the chicks were biased against yellow, preferring green crumbs (Treatment 1); however, in Treatment 2, when the chicks were familiar
with both the yellow and the green crumbs, they were biased against the green crumbs, preferring to eat yellow (Dunn’s post-hoc test Treatment 1 vs. 2 p < 0.05). The addition of pyrazine odour did not change the eating bias towards the novel or familiar yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 3 NS; Treatment 2 vs. 4 NS respectively). In contrast to these derived results, if the number of yellow and green crumbs eaten in the first trial is examined (Fig. 3.1.3), it becomes clear that when both the yellow and the pyrazine odour were novel (Treatment 3) the chicks decreased their willingness to eat the green (Dunn’s post-hoc test Treatment 1 vs. 3 p < 0.05) as well as the yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 3 p < 0.05). Therefore, even though the chicks ate fewer yellow crumbs in Treatment 3 than in Treatment 1, the eating bias for these two treatments was the same. This suggests that the pyrazine odour did have an effect on the chicks’ willingness to eat the yellow crumbs; however, this was masked in the analysis of eating bias.
Figure 3.1.4 – The eating bias (±s.e.) (number of green minus number of yellow crumbs eaten) in the first trial across the six treatment groups.

When the chicks were familiar with the pyrazine odour (Treatments 5 and 6), the eating bias (Fig. 3.1.4) against yellow was increased (Dunn’s post-hoc test Treatment 1 vs. 5 p < 0.05). The method of familiarisation with the odour had no effect on this (Dunn’s post-hoc test Treatment 5 vs. 6 NS). The eating bias in Treatment 5 was significantly higher than in Treatment 3 (Dunn’s post-hoc test Treatment 3 vs. 5 p < 0.05), which suggests that chicks familiar with pyrazine were more biased against yellow than chicks for whom pyrazine was novel. However, if we examine the actual number of crumbs eaten (Fig 3.1.3) we can see that there was no significant difference between the number of yellow crumbs eaten by chicks in Treatments 3 and 5 (Dunn’s
post-hoc test Treatment 3 vs. 5 NS), so the cause of the difference in the eating bias lies in the number of green crumbs eaten (Dunn’s post-hoc test Treatment 3 vs. 5 p < 0.01).

When the chicks were familiar with pyrazine they avoided the yellow crumbs but continued to attack the green crumbs; however, when the odour was novel they avoided both the yellow and green crumbs. It is this difference in behaviour that caused the eating bias difference between Treatments 3 and 5.

3.1.4 – DISCUSSION

Novel yellow prey elicited little neophobic response, which is in keeping with the findings of Roper and Cook (1989). However, the results show that the chicks exhibited significant levels of dietary conservatism towards the yellow signal. This difference in the two behavioural responses to the same signal adds weight to the suggestion that they are two distinct behavioural processes involved in innate avoidance (Marples and Kelly 1999).

The addition of novel pyrazine odour to novel yellow crumbs prolonged both neophobia and dietary conservatism. Kelly and Marples (2004) noted that the addition of a novel odour (almond or vanilla) to novel colours which were not classically associated with warning displays (blue or green) enhanced dietary conservatism but not neophobia. The present results suggest that this does not hold true where warning signals are concerned, as both processes of innate avoidance behaviour towards yellow were enhanced by the presence of the pyrazine odour.

The chicks showed very little dietary conservatism towards the familiar yellow crumbs which suggests that familiarity with a colour deactivates the dietary conservatism response towards it. The addition of novel pyrazine odour to the familiar yellow crumbs did not reinstate the neophobia or dietary conservatism. Rowe and
Guilford (1999a) noted that although pyrazine odour could reactivate avoidance towards familiar yellow prey, this ability diminished as the chicks became more familiar with the yellow crumbs. The chicks in this current study were very familiar with yellow crumbs, and although they showed no evidence of neophobia towards familiar yellow crumbs in the presence of pyrazine odour, they showed a non-significant increase in dietary conservatism. This suggests that dietary conservatism is not only a more prolonged process, but is also more resistant to deactivation through familiarity, which is in keeping with the findings of Marples et al. (2007). This difference in the effect of the multimodal signal on neophobia and dietary conservatism further highlights the need to differentiate between these two processes when studying innate avoidance behaviour. Neophobia is such a short-lived response that the difference between the groups tells us little about the chicks' real innate avoidance of the crumbs. The dietary conservatism data provides a more solid picture of their willingness to accept the food into the diet, and therefore may be a preferable measure.

The results from this experiment also demonstrate that pyrazine odour increased neophobia and dietary conservatism towards novel yellow prey regardless of whether the odour was novel or familiar to the chicks. This is contrary to the results of Rowe and Guilford (1999a), who reported that novel, but not familiar, pyrazine enhanced attack bias against yellow crumbs, and to Jetz et al. (2001) who reported a similar result with familiar ethyl acetate. The chicks in our experiment were more familiar with pyrazine than those in the other studies, as they encountered 24 crumbs that smelt of pyrazine during each of the 1-minute training trials, as well as experiencing a 40-minute social training session. In contrast, the chicks in the Rowe and Guilford (1999a) experiment received three training trials during which they encountered eight crumbs that smelt of pyrazine. Despite this greater familiarity of the chicks with the
pyrazine odour, a positive effect of the odour was still detected. Possible reasons for
the disparity of these results are discussed further in Section 3.3.

Rowe and Guilford (1996, 1999a, b) used attack bias, the number of green
crums eaten minus the number of yellow crumbs eaten, as a measure of innate
avoidance. When the analogous measure, eating bias, was investigated for this current
experiment, the results still contradicted those of Rowe and Guilford (1999a) and
showed that the chicks responded to novel and familiar pyrazine odour in a similar
manner. The data analysis of this current experiment suggests that the derived measure
of attack bias may hide subtle behavioural effects. Therefore latencies to overcome
neophobia and dietary conservatism, and the number of crumbs eaten may be more
reliable measures of innate avoidance behaviour.

The chicks' responses to the green crumbs in this experiment were somewhat
unexpected. Even though the chicks were familiar with green food, they still seemed to
express a high level of wariness towards the green crumbs. When both yellow and
green were familiar the chicks showed an eating bias against green. This suggests that
this shade of green may have been viewed as aversive by the chicks. There are
examples of green being used as a warning colour by defended insects, such as the
green metallic weevil (*Phyllobius urtica*) (Rothschild and Moore 1987). Therefore, a
paler, less intrusive green was used for subsequent experiments. Also, the chicks in
this experiment only encountered the green crumbs in the four training trials and the
social training, rather than receiving it during pre-training and at home as in subsequent
experiments. They therefore may not have viewed the green crumbs as familiar,
alternative prey. However, it is also possible that the chicks avoided the familiar green
crumbs because of their proximity to the warningly coloured crumbs in a manner
similar to that observed by Roper (1993) and Mappes *et al.* (1999).
3.2 - EXPERIMENT 2 - The effect of pyrazine odour on neophobia and dietary conservatism towards red crumbs.

3.2.1 - INTRODUCTION

Red, as well as yellow, is frequently used as a warning colour by defended insect species (Cott 1940; Sillen-Tullberg 1985a). Many defended red insects also use pyrazine as a warning odour (Moore et al. 1990). Rowe (1998) observed that chicks showed a similar innate avoidance response towards red crumbs paired with the pyrazine odour as they did towards yellow crumbs paired with pyrazine. This experiment sought to investigate this further, while examining innate avoidance behaviour as the two distinct processes of neophobia and dietary conservatism (Marples and Kelly 1999).

3.2.2 – METHODS

The experiment was conducted over two weeks, with 31 chicks tested in week one and 29 in week two. All treatments were tested during both weeks. Mann-Whitney U tests showed that there were no significant differences between the data collected over the two weeks, and therefore, the data were combined. This gave a total of 14 replicates in Treatment 1, 16 replicates in Treatment 2 and 15 replicates in Treatments 3 and 4. The method used was similar to that described for Experiment 1 (Section 3.2.1), except that a paler green was used, and the chicks were given green food at home, and were pre-trained using green food on day one. The chicks were also given red crumbs rather than yellow crumbs during the test trials (Table 3.2).
Table 3.2: Treatments received during the training and test trials, indicated as crumb colours and odours. All crumbs were palatable.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Training food</th>
<th>Test food</th>
<th>Tests for response to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green</td>
<td>Green; red</td>
<td>Novel red</td>
</tr>
<tr>
<td>2</td>
<td>Green; red</td>
<td>Green; red</td>
<td>Familiar red</td>
</tr>
<tr>
<td>3</td>
<td>Green</td>
<td>Green; red plus pyrazine</td>
<td>Novel red plus novel pyrazine</td>
</tr>
<tr>
<td>4</td>
<td>Green; red</td>
<td>Green; red plus pyrazine</td>
<td>Familiar red plus novel pyrazine</td>
</tr>
</tbody>
</table>

3.2.3 – RESULTS

There were significant differences in the time taken to overcome neophobia towards red crumbs across the groups (Kruskal-Wallis test, $\chi^2 = 12.096$, d.f. = 3, $p < 0.01$, Fig. 3.2.1). Familiarity with red did not decrease neophobia, as there was no difference in the time taken to overcome neophobia by chicks in Treatments 1 and 2 (Dunn’s post-hoc test Treatment 1 vs. 2 NS). The addition of pyrazine significantly prolonged neophobia towards novel red crumbs, as chicks in Treatment 3 took significantly longer to overcome neophobia than those in Treatment 1 (Dunn’s post-hoc test Treatment 1 vs. 3 $p < 0.01$). Despite the graph’s appearance, this effect was not significant when the chicks were familiar with red, as there was no significant difference between the time to overcome neophobia between chicks in Treatments 2 and 4 (Dunn’s post-hoc test Treatment 2 vs. 4 NS). Chicks did, however, take significantly longer to overcome neophobia of familiar red crumbs that smelt of pyrazine than odourless, novel red crumbs (Dunn’s post-hoc test Treatment 1 vs. 4 $p < 0.01$). There was no significant difference in the time taken to overcome neophobia of novel compared to familiar red crumbs when both smelt of pyrazine (Dunn’s post-hoc test Treatment 3 vs. 4 NS). There were also no significant differences in the times
taken to overcome neophobia of green crumbs across the groups (Kruskal-Wallis test, $\chi^2 = 4.853$, d.f. = 3, NS, Fig. 3.2.1).

When the latency to peck the first green and the first red crumb was compared for each treatment, there were no significant differences. From the graph (Fig. 3.2.1) the chicks appeared to take longer to peck red rather than green crumbs when the red crumbs smelt of pyrazine, but this was not significant for either the novel or familiar red treatments. When the red crumbs were odourless there was no difference in the time taken to peck red or green crumbs, and familiarity with the red crumbs did not change this result.

![Figure 3.2.1: The mean latency (±s.e) (in seconds) to overcome neophobia of green and red crumbs (indicated by the colour of the bars) in the presence and absence of pyrazine. Treatment 1 n = 14, Treatment 2 n = 16 and Treatments 3 and 4 n = 15.](image)

When dietary conservatism was measured, the results followed a similar pattern to those observed with neophobia. There were significant differences in the time taken...
to overcome dietary conservatism of red across the groups (Kruskal-Wallis test, \(\chi^2 = 11.335, \text{d.f.} = 3, \ p < 0.01, \text{Fig. 3.2.2}\)). The addition of pyrazine prolonged dietary conservatism of novel red, as chicks in Treatment 3 took significantly longer to overcome dietary conservatism than chicks in Treatment 1 (Dunn’s post-hoc test Treatment 1 vs. 3 \(p < 0.01\)). Familiarisation of the chicks with red crumbs did not reduce dietary conservatism, as there was no difference in the time taken to overcome dietary conservatism between the novel and familiar red crumbs (Dunn’s post-hoc test Treatment 1 vs. 2 NS). The addition of pyrazine to familiar red crumbs appeared to increase dietary conservatism, although this was a non-significant trend (Dunn’s post-hoc test Treatment 2 vs. 4, critical value = 11.99, mean rank difference = 10.07). This suggests that familiarisation with the colour component of the signal may alter the interaction of the colour and odour cues. However, there was no significant difference in the dietary conservatism exhibited towards the novel red crumbs that smelt of pyrazine and the familiar red crumbs that smelt of pyrazine (Dunn’s post-hoc test Treatments 3 vs. 4 NS).

There were no significant differences in the time taken to overcome dietary conservatism of green across the groups (Kruskal-Wallis test, \(\chi^2 = 3.639, \text{d.f.} = 3, \ NS\)). Pyrazine did not make the chicks more dietarily conservative towards the red than the green crumbs. From the graph (Fig. 3.2.2) it seems that chicks in treatments exposed to pyrazine (Treatments 3 and 4) showed prolonged dietary conservatism towards green crumbs as well as red crumbs, although this change was not significant for the green crumbs.
Figure 3.2.2: The mean latency (±s.e) (in seconds) to overcome dietary conservatism of green and red crumbs (indicated by the colour of the bars) in the presence and absence of pyrazine. Treatment 1 n = 14, Treatment 2 n = 16 and Treatment 3 and 4 n = 15.

When the number of crumbs eaten rather than the latency to eat was examined, it was noted that there were significant differences in the number of red crumbs eaten during the first trial (Kruskal-Wallis test, $\chi^2 = 6.576$, d.f. = 3, $p < 0.05$, Fig. 3.2.3). Chicks offered novel red crumbs that smelt of pyrazine ate significantly fewer red crumbs than chicks offered odourless novel red crumbs (Dunn’s post-hoc test Treatment 1 vs. 3 $p < 0.05$). However, there were no significant differences between the number of familiar red crumbs eaten in the presence or absence of the pyrazine odour (Dunn’s post-hoc test Treatment 2 vs. 4 NS). There were also no significant differences in the number of green crumbs eaten across the four treatments (Kruskal-Wallis test, $\chi^2 = 2.748$, d.f. = 3, NS, Fig. 3.2.3). Chicks in Treatments 2 and 3 ate
significantly more green than red crumbs in the first trial (Mann-Whitney U test, Treatment 2 $U = 66.50, n = 16, 16, p < 0.05$; Treatment 3 $U = 53.50, n = 15, 15, p < 0.05$; Fig. 3.2.3).

The chicks showed different eating biases across the four treatments (Kruskal-Wallis test, $\chi^2 = 8.341, df. = 3, p < 0.05$, Fig. 3.2.4). The addition of pyrazine to novel red crumbs (Treatment 3) produced the only significant difference in the eating biases (Dunn’s post-hoc test Treatment 1 vs. 3 $p < 0.05$), increasing the bias against red crumbs. From the graph (Fig. 3.2.4) it appears that the chicks had a greater bias against odourless, familiar red crumbs than odourless, novel red crumbs, and a greater bias against novel red crumbs that smelt of pyrazine than familiar red crumbs that smelt of pyrazine. However, these were non-significant trends (Dunn’s post-hoc test Treatment
1 vs. 2, critical value = 12.45, mean rank difference = 11.65; Treatment 3 vs. 4, critical value = 12.42, mean rank difference = 12.16).

![Graph](image)

**Figure 3.2.4:** The eating bias (±s.e) (number of green minus number of red crumbs eaten) in the first trial across the four treatment groups.

When this graph (Fig. 3.2.4) was interpreted using the number of crumbs attacked (Fig. 3.2.3) it was apparent that the large increase in the eating bias of chicks in Treatment 3 was due to a decrease in the number of red crumbs attacked, as well as an increase in the number of green crumbs attacked when compared to the eating bias of chicks in Treatment 1. Ultimately, however, these changes in the number of crumbs eaten were non-significant. The non-significant increase in eating bias against familiar versus novel red crumbs from Fig. 3.2.4 may also be explained by referring to Fig. 3.2.3. When the red crumbs were familiar (Treatment 2), the chicks appeared to eat slightly fewer than when red was novel to them (Treatment 1). Chicks in Treatment 2
also appeared to increase the number of green crumbs that they ate. This may have caused the apparent increase in bias between Treatments 1 and 2 in Fig. 3.2.4. A similar analysis can be applied to the difference in eating bias between Treatments 3 and 4. When the chicks were offered novel red crumbs that smelt of pyrazine, they ate many green crumbs and relatively few red crumbs. However, when offered familiar red crumbs that smelt of pyrazine, they ate fewer green crumbs and slightly fewer red crumbs, which explains the change in bias observed in Fig. 3.2.4.

3.2.4 – DISCUSSION

In a similar manner to that observed with yellow crumbs in Experiment 1, the addition of novel pyrazine odour to novel red crumbs prolonged both neophobia and dietary conservatism. Kelly and Marples (2004) observed that pyrazine odour enhanced dietary conservatism by zebra finches towards novel red prey but did not affect neophobia. This may reflect the difference between behaviour observed with domestic chicks in the laboratory and that of captive adult passerines.

When compared to familiar red crumbs, there were no differences in either neophobia or dietary conservatism towards novel red crumbs in the presence or absence of pyrazine, as there had been in response to the yellow crumbs in Experiment 1. This suggests that the familiarisation process with red may have been ineffective. It may be that the chicks needed more experience with red than yellow in order to deactivate their innate avoidance behaviour, suggesting that red may be a more salient signal than yellow.

Although familiarity with red crumbs did not reduce either neophobia or dietary conservatism in Experiment 2, it would appear that familiarity with the colour
component of the signal affected how the colour and odour cues of the multimodal
display interacted. The addition of pyrazine to novel red crumbs prolonged both
neophobia and dietary conservatism when compared to odourless novel red crumbs;
however, when the red crumbs were familiar, this increase was reduced and the results
were no longer significant.

In this experiment the number of crumbs of each colour eaten, and the eating
bias, reflected the same pattern of results as the latency measure. They confirm that the
addition of pyrazine to a novel red signal increased innate avoidance behaviour, but
had no effect when red was familiar to the chicks.

Paler green crumbs were used in this experiment than in Experiment 1, and the
results from Experiment 2 suggest that the chicks were less wary of these paler green
crumbs. There were no significant differences between the neophobia and dietary
conservatism showed towards the red and green crumbs in any of the treatments. This
suggests that the chicks treated the green and red crumbs in the same manner, which
supports Mappes et al.'s (1999) suggestion that palatable insects may gain protection
from living in close proximity to defended insects. However, in Experiment 1
neophobia and dietary conservatism of the green crumbs fluctuated across the
treatments, whereas no significant fluctuation was observed in Experiment 2. Also
when both red and green were familiar to the chicks they showed an eating bias against
red, whereas when yellow and green were familiar they showed a bias against green.
This suggests that the pale green used in Experiment 2 may be less aversive to the
chicks and therefore a more preferable alternative food type for innate avoidance
experiments.
The results from Experiments 1 and 2 clearly show that pyrazine odour enhances both neophobia and dietary conservatism of novel yellow and red crumbs. If these results reflect how wild birds respond towards insect prey then an insect that had both a yellow or red visual signal and pyrazine odour would be better protected through both processes of avian predator innate avoidance behaviour than an insect that signalled using a visual display alone.

Familiarity with the odour appeared to have no effect on how the odour enhanced innate avoidance of the yellow crumbs in Experiment 1, which is contrary to the findings of Rowe and Guilford (1999a). Rowe and Guilford (1999a) put the pyrazine odour beneath both the green and yellow crumbs so it therefore acted as a background odour and not an additional discriminatory cue as in Experiments 1 and 2. The novel pyrazine reduced the number of both green and yellow crumbs eaten in Rowe and Guilford’s (1999a) experiment but caused a greater reduction in the number of yellow than green crumbs consumed. When the pyrazine was familiar, Rowe and Guilford (1999a) observed no such avoidance of the yellow crumbs. If anything, the presence of the familiar pyrazine beneath both the green and yellow crumbs appeared to increase the chicks’ willingness to eat both colours of crumb. It may have been because the pyrazine odour was beneath all the crumbs, and was familiar, that the chicks paid no attention to the odour. Jetz et al. (2001) also placed the odour beneath every crumb and recorded similar results to Rowe and Guilford (1999a) with familiar ethyl acetate odour. Rowe and Guilford (1999a) had a sample size of eight chicks per treatment, while Jetz et al. (2001) had a sample size of between 11 and 18 depending on the treatment. As these samples size are similar to the current experiments it would
suggest that the disparity in the result between these experiments is due to methodical
differences rather than statistical power.

In contrast, pyrazine odour was placed solely beneath the aposematically
coloured crumbs in Experiments 1 and 2, and therefore may have acted as an additional
discriminatory cue between the green and aposematically coloured crumbs. This may
explain the ability of the familiar pyrazine odour to enhance innate avoidance
behaviour. Both Marples and Roper (1997) and Rowe and Guilford (1999a) suggest
that the differences in responses towards odours observed from laboratory experiments
may be due to differences in odour intensity; however, the concentration of the
pyrazine solution used in this and the Rowe and Guilford (1999a) experiment were
identical.

It is also possible that the lack of effect of the familiar pyrazine may have been
because Rowe and Guilford's (1999a) method of odour familiarisation could have
caused the chicks to form a positive association between the pyrazine odour and
palatable food. However, in Experiment 1 it was noted that the manner with which the
chicks were made familiar with the pyrazine odour did not significantly affect the
results. Guilford et al. (1987) showed that chicks can use pyrazine odour as a
discriminatory learning cue when presented with an avoidance learning task, but they
quickly forget this learned avoidance within 24 hours. It may be that although the
chicks had been familiarised with the pyrazine odour they did not remember its
consequences 24 hours later and therefore responded to novel and familiar pyrazine in
a similar manner. The ability of familiar odour but not familiar colour to elicit innate
avoidance in a multimodal signal may also be a reflection of the fact that vision is the
dominant sense in birds (Verheyden and Jouventin 1994, Gill 1994), so familiarity with
a visual cue may have a greater impact on behavioural responses than familiarity with
an olfactory cue.
The chicks appeared to be more resistant to familiarisation with red than yellow crumbs. Familiarisation with red did not decrease either neophobia or dietary conservatism, whereas the same number of training trials was sufficient to deactivate both processes of innate avoidance behaviour towards yellow crumbs. Although the chicks in Experiment 2 appeared to be unfamiliar with the red crumbs, there were no significant difference in the neophobia and dietary conservatism towards odourless familiar red crumbs and familiar red crumbs that smelt of pyrazine, which reflects the results from Experiment 1.

Chicks showed less neophobia and dietary conservatism towards familiar yellow crumbs that smelt of pyrazine, than novel crumbs that smelt of pyrazine. On the other hand neophobia or dietary conservatism was the same towards novel red crumbs that smelt of pyrazine and familiar red crumbs that smelt of pyrazine. This all suggests that the chicks are much more resistant to familiarisation with red than yellow crumbs. This may be because red is a more salient cue than yellow and therefore the birds need to be more familiar with it before their innate avoidance is deactivated. It is also possible that as the chicks in Experiment 2 were much more familiar with the green crumbs and were given a less intense green colour, they may have responded differently to the familiar red crumbs.

Rothschild (1984) noted that in order for birds to be discerning about their foraging choices, alternative prey types need to be available. It has been suggested that in order for birds to show dietary conservatism, they need to have alternative familiar prey present (Thomas et al. 2003; Kelly and Marples 2004). In Experiments 1 and 2 of this chapter familiar green food was the alternative prey type, whereas Rowe and Guilford (1999a) presented the chicks with novel yellow and green prey, so neither was familiar to the birds. This lack of an alternative, familiar prey may have altered the chicks’ response to the novel yellow crumbs and reduced their ability to show
wariness, which may further explain why familiar pyrazine failed to enhance innate avoidance in Rowe and Guilford’s (1999a) experiment.

The results from Experiments 1 and 2 suggest that it is important to differentiate between the two aspects of innate avoidance behaviour when conducting research of this kind, as the signals tested often had different effects on neophobia and dietary conservatism. It is also important to note that the chicks in Experiment 2 showed neophobia towards red crumbs that smelt of pyrazine, but zebra finches do not (Kelly and Marples 2004). This suggests that dietary conservatism may be a more consistent and reliable measure of innate avoidance behaviour, and therefore a preferable measure to neophobia. Birds often make quick foraging decisions and may attack prey without much apparent deliberation (Rothschild 1984), therefore rendering neophobia a poor measure of decision making. Dietary conservatism, on the other hand, requires the birds to have eaten three individuals in one trial (Marples et al. 2007) and therefore reflects a greater acceptance of the prey item, and allows time for cognitive processes to occur. Guilford (1986) proposed the distance detection hypothesis, which stated that the longer a bird has to look at a prey individual before attacking; the more likely it is that the bird’s reaction to the prey is to reflect its true preferences. This longer time period may afford the bird a greater opportunity to recognise the prey as a potentially defended food item. This then adds further weight to the argument that dietary conservatism is a more reliable measure of innate avoidance than neophobia.

A comparison of the different measures of innate avoidance behaviour provides some important conclusions. Many previous workers have used the number of prey items attacked (Marples and Roper 1997) or attack bias (Guilford and Rowe 1996, 1999a, b; Jetz et al. 2001) as measures of innate avoidance behaviour. The comparison of the eating bias measure and the actual number of crumbs eaten suggests that the
eating bias may mask true behaviour, as was seen in Experiment 1 of this chapter. Jetz et al. (2001) noted that latencies may offer a more sensitive picture of behavioural choices and one that picks up more subtle predatory decisions than a measure of the number of crumbs eaten. Birds may choose to eat the same number of prey but may take longer to do it in one treatment than another. This adds weight to the argument against using derived measures, and suggests that empirical measures provide a clearer picture of behaviour.

The chicks' responses to the familiar green crumbs were of interest. The high level of avoidance of familiar green crumbs in Experiment 1 led to the use of paler green in the subsequent experiments, and greater exposure to them. This created an environment in which dietary conservatism towards novel foods was more likely to be observed (Thomas et al. 2004). The chicks in both Experiment 1 and 2 tended to show the same level of neophobia and dietary conservatism towards the aposematically coloured crumbs, and the green crumbs. This supports the suggestion that palatable prey may gain protection from close proximity to defended prey species (Mappes et al. 1999). However, in Experiment 1 the response to green fluctuated across the treatments, more than in Experiment 2. Also when the chicks were familiar with both yellow and green crumbs in Experiment 1, they showed significantly more dietary conservatism towards the green than yellow crumbs, and an eating bias against green crumbs. Whereas in Experiment 2 there was no significant difference in the dietary conservatism showed towards the familiar red and green crumbs and the chicks showed an eating bias against red, which supports the suggestion that the paler green was a preferable control food.
CHAPTER 4

The effect of buzzing of B. terrestris on innate avoidance behaviour towards aposematically coloured food

Acknowledgement and authorship

Both experiments reported in this chapter were designed, executed and analysed by E. Siddall. Dr. Marples and Dr. Thomas contributed to the design of the experiments. Experiment 3 is part of the manuscript "Hear no evil: The effect of auditory warning signals on avian innate avoidance, learned avoidance and memory" which is in preparation for submission to Behavioral Ecology.
4.1 - EXPERIMENT 3 - The effect of the buzzing of *B. terrestris* on neophobia and dietary conservatism towards yellow and red crumbs

4.1.1 – INTRODUCTION

The buzzing produced by hymenopteran species has been suggested to be an auditory warning display (Gaul 1952, Haskell 1961). Rowe and Guilford (1999b) showed that the agitated buzzing of *B. terrestris* could enhance innate avoidance of novel green and warningly coloured novel yellow crumbs. They hypothesised that innate avoidance behaviour may be enhanced by any auditory stimulus as long as it is novel, in a similar manner to the behavioural changes observed in the presence of pyrazine. However, Hauglund *et al.* (2006) observed no effect of the flying buzzing of a wasp (*D. media*) on the mean avoidance of novel yellow prey played back to chicks at 66-72 dB. The volume used by Hauglund *et al.* (2006) was clearly detectable by newly hatched chicks (Saunders and Salvi 1993, Dooling 2004). Rowe and Guilford (1999b) did not report the volume of their auditory signal, but it was clearly audible to the experimenter (C. Rowe pers. comm.).

The disparity between the Rowe and Guilford (1999b) and Hauglund *et al.* (2006) results may be due to several factors. Firstly, the two experiments used different types of buzzing. Rowe and Guilford (1999b) used the agitated buzzing of *B. terrestris* trapped in a net, whereas Hauglund *et al.* (2006) used the flying buzzing of *D. media*. The buzzing of a flying insect is a by-product of its flight and is not produced in response to a threatening situation (Otis 2005). Warning sounds and odours tend not to be displayed continuously but are produced in response to a threat (Rowe 2002; Brown *et al.* 2007). Therefore, it may be that the buzzing sound of flight does not operate as a warning sound, whereas agitated buzzing does. Secondly,
Hauglund et al. (2006) used unpalatable rather than palatable prey, which may have altered the results as the chicks' responses may have been due to learned avoidance during the first trial, rather than to innate avoidance. Finally, Hauglund et al. (2006) used 96-day old chicks, whereas Rowe and Guilford (1999b) used day-old chicks. Saunders and Salvi (1993) noted that newly-hatched chicks have a well developed sense of hearing that becomes more acute within the first few weeks of life. However, older chicks often show extensive hearing damage from exposure to the loud environment of commercial hatcheries (Durham et al. 2002). It is therefore possible that the chicks Hauglund et al. (2006) used in their study had acquired damage to their hearing.

The experiment presented below aimed to address the disparity between the results of Rowe and Guilford's (1999b) and the Hauglund et al. (2006) experiments, and to investigate the effect of agitated buzzing of *B. terrestris* on the two distinct processes of innate avoidance, neophobia and dietary conservatism (Marples and Kelly 1999). This was the first time such an investigation had been undertaken.

4.1.2 – METHODS

The experiment was conducted over one week with 14 chicks in each treatment. The method used was similar to that described for Experiment 2 (Section 3.2.2), except that in this experiment buzzing was the additional signal in place of the pyrazine, and in Treatments 1 and 3 the novel crumbs were yellow (Table 4.1). The agitated buzzing sound of *B. terrestris* was played at between 65-70 dB, similar to the level used by Hauglund et al. (2006) (see Section 2.3). At this volume the buzzing was clearly
audible to the experimenter, and therefore possibly of a similar level to that used by Rowe and Guilford (1999b).

Table 4.1: Treatments used during the training and test trials, indicated as crumb colours and odours. All crumbs were palatable. The buzzing sound was played as a chick approached either a red or yellow crumb.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Training food</th>
<th>Test food</th>
<th>Test for response to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green</td>
<td>Green; yellow</td>
<td>Novel yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green</td>
<td>Green; red</td>
<td>Novel red</td>
</tr>
<tr>
<td>3</td>
<td>Green</td>
<td>Green; yellow plus buzzing</td>
<td>Novel yellow plus novel buzzing</td>
</tr>
<tr>
<td>4</td>
<td>Green</td>
<td>Green; red plus buzzing</td>
<td>Novel red plus novel buzzing</td>
</tr>
</tbody>
</table>

4.1.3 – RESULTS

The agitated buzzing sound appeared to have no effect on how long the chicks took to overcome their neophobia of either yellow or red crumbs. There were no significant differences between the four treatments in the time taken to overcome neophobia towards the aposemitically coloured crumbs (Kruskal-Wallis test, $\chi^2 = 4.905$, $d.f. = 3$, NS, Fig. 4.1.1). Similarly, when the responses to the two aposematic colours were compared, there was no difference in the time taken to overcome neophobia of yellow or red crumbs, suggesting that both colours were equally effective as warning colours. The association of the green crumbs with the various novel signals had no significant effect on how quickly the chicks pecked at them (Kruskal-Wallis test, $\chi^2 = 7.166$, $d.f. = 3$, NS, Fig. 4.1.1). There were no significant differences in the time the chicks took to peck their first crumb of any colour in any of the treatments (Fig. 4.1.1), which suggests that neophobia towards the novel aposematic colours was
no greater than towards the familiar green crumbs, although from the graph the green crumbs appeared to be avoided more than the aposematically coloured crumbs.

Figure 4.1.1: The mean latency (±s.e) (in seconds) to overcome neophobia of the familiar green and novel aposematically coloured crumbs (indicated by the colour of the bars) in the presence and absence of buzzing accompanying the aposematic coloured crumbs. There were 14 replicates in each of the four treatments.

The addition of buzzing had no effect on the dietary conservatism exhibited towards either red or yellow crumbs. There was no significant difference in the latency to overcome dietary conservatism towards any of the aposematically coloured crumbs, either in the presence or absence of the buzzing sounds (Kruskal-Wallis test, $\chi^2 = 1.084$, $d.f. = 3$, NS, Fig. 4.1.2). As with the neophobia data, this suggests that both yellow and red are equally effective warning colours.

The association of the green crumbs with the various warning signals had no significant effect on how quickly the chicks ate them (Kruskal-Wallis test, $\chi^2 = 1.028$, $d.f. = 3$, NS, Fig. 4.1.2). In Treatments 1, 2 and 3 the familiar green crumbs were eaten
as quickly as the novel aposematic crumbs in each treatment. In Treatment 4, however, the chicks took significantly longer to overcome dietary conservatism of novel red crumbs in the presence of the buzzing sounds than familiar green crumbs (Mann-Whitney U test, $U = 47.00$, $n = 14, 14$, $p < 0.05$, Fig. 4.1.2).

**Figure 4.1.2:** The mean latency (±s.e) (in seconds) to overcome dietary conservatism of familiar green and the novel aposematically coloured crumbs (indicated by the colour of the bars) in the presence and absence of buzzing accompanying the aposematic coloured crumbs.

There were no significant differences between the treatments in the number of crumbs of any one colour eaten in the first trial (Kruskal-Wallis test, aposematically coloured crumbs $\chi^2 = 1.431$, $d.f. = 3$, NS; green crumbs $\chi^2 = 5.589$, $d.f. = 3$, NS Fig. 4.1.3). There were also no significant differences between the number of green and yellow crumbs eaten by chicks in either Treatment 1 (Mann-Whitney U test, $U = 85.00$, $n = 14, 14$, NS) or Treatment 3 (Mann-Whitney U test, $U = 64.50$, $n = 14, 14$, NS). However, a non-significant trend suggests that chicks in Treatment 2 ate more green
than red crumbs (Mann-Whitney U test, $U = 56.50$, $n=14, 14$, $p = 0.056$), and when buzzing was added to the red crumbs in Treatment 4 the chicks ate significantly more green than red crumbs (Mann-Whitney U test, $U = 37.00$, $n=14, 14$, $p < 0.01$).

**Figure 4.1.3:** The mean number (±s.e) of familiar green and novel (yellow or red) crumbs (indicated by the colour of the bars) eaten during the first trial in the presence or absence of the buzzing sound of *B. terrestris*.

The eating bias (Fig. 4.1.4) did not differ significantly across the four treatment groups (Kruskal-Wallis test, $\chi^2 = 6.871$, $d.f. = 3$, NS), and any trends suggested by the graph are attributable to differences in consumption of the green, not the aposematically coloured crumbs. However, the non-significant trend in the graph suggests that the addition of buzzing to the yellow crumbs may have increased the bias against yellow. When we consider the number of crumbs eaten in the first trial (Fig. 4.1.3), it is apparent that the increase in eating bias with buzzing yellow was due not to
a decrease in the number of yellow crumbs eaten in the presence of buzzing, but rather an increase in the number of green crumbs eaten.

**Figure 4.1.4:** The eating bias (±s.e) (number of green crumbs eaten minus number of coloured crumbs eaten) in the first trial across the four treatment groups.

The graph (Fig. 4.1.4) also suggests that red may be a more effective warning colour than yellow as the eating biases against red were greater than those against yellow crumbs, although this was not significant. However, once again, this was due to differences in the consumption of green crumbs (Fig. 4.1.3). Chicks in the treatments offered red crumbs (Treatments 2 and 4) appeared to eat more green crumbs in the first trial than chicks in treatments offered yellow crumbs (Treatments 1 and 3). This suggests that the chicks may have been better able to discriminate between red and green crumbs than between yellow and green crumbs.
4.1.4 – DISCUSSION

The results showed that buzzing did not increase either neophobia or dietary conservatism towards yellow or red crumbs. This is contrary to Rowe and Guilford’s (1999b) results, which suggested that buzzing increased innate bias against yellow prey, but is in keeping with Hauglund et al.’s (2006) result. It would appear that the chicks could hear the buzzing sound as they often raised their heads and looked around when it was played, suggesting that even though they could hear the sound, it had no effect on their innate avoidance behaviour.

Rowe and Guilford (1999b) played the buzzing sound continuously while the chick was in the experimental arena, whereas in this present study, and the Hauglund et al. (2006) study, the buzzing sound was played only when the chick approached an aposematically coloured crumb. The differences in the methodology of sound presentation may explain the difference between Rowe and Guilford’s (1999b) results, and those observed both in this current experiment and by Hauglund et al. (2006). Insects tend to produce warning sounds when approached or attacked by a predator (Rowe 2002, Brown et al. 2007). Therefore, the method of sound presentation used in this and the Hauglund et al. (2006) experiment more closely resembles how insects display their warning signals, with the visual component of the display being presented first followed by the auditory component. The continuous buzzing sound used by Rowe and Guilford (1999b) may have made the chicks abnormally wary of any novel or warningly coloured prey, thus making them willing to eat only familiar food. Rowe and Guilford (1999b) had a sample size of 12 chicks per treatment, while Hauglund et al. (2006) had a sample size of eight chicks per treatment.

As suggested in Section 3.3, the use of eating bias as a measure of innate avoidance behaviour may mask how the chicks are truly responding to the food offered.
to them. Though non-significant, our results suggest that the chicks may have shown an increased eating bias against buzzing yellow crumbs when compared to silent yellow crumbs. However, this apparent difference was due to an increase in the number of green crumbs eaten in the presence of buzzing rather than to a decrease in the number of yellow crumbs attacked. This aspect of the chicks’ behaviour was not apparent from the eating bias measure alone. Rowe and Guilford (1999b) also reported the mean number of crumbs of each colour eaten and the standard error. These data suggest that in their study the attack bias measure accurately represented the behaviour of their chicks as they showed that an increased bias against a particular colour was due to an increased avoidance of that colour of crumbs, rather than to an increased acceptance of the familiar coloured food. However, Rowe and Guilford (1999b) did not provide further analysis of these data.

When the number of crumbs eaten was examined, it was noted that the chicks did not appear to differentiate between either the yellow and green crumbs, or between the red and green crumbs. However, the addition of buzzing to the red crumbs appeared to enable the chicks to distinguish between the green and red crumbs. The sound was played before the chicks attacked a crumb, and therefore may have acted as an additional discriminatory cue. Alternatively, as Rothschild et al. (1984) suggested, buzzing may operate as an alerting signal that enables predators to differentiate between prey. If so, buzzing may help to improve discrimination in a similar manner to that observed with pyrazine (Experiment 1). Hauglund et al. (2006) also noted that buzzing may have discrimination enhancing effects, so although buzzing does not appear to enhance innate avoidance behaviour, it may help to improve discrimination between prey types.

Rowe and Guilford (1999b) suggest that any novel sounds may enhance innate avoidance behaviour, but Hauglund et al. (2006) failed to find an effect of flying
buzzing on innate avoidance behaviour. This suggests that there might be something special about agitated buzzing as an auditory signal. However, the results from this current experiment conclude that agitated buzzing, at least when presented in this manner, does not have any effect on innate avoidance behaviour. It may be that buzzing does not operate as a warning signal for avian predators but may be an effective warning signal against other species of predators, such as spiders (Myers 1935) or toads (Brower and Brower 1961). Buzzing may also operate as an intraspecific warning signal (Kirchner and Roschard 1999), which may explain its lack of effect on avian predatory decisions. However, other insect sounds may operate as warning signals to predators, and this area warrants further research.

This experiment also provided an additional result. It would appear that chicks consider red and yellow to be as effective as each other as warning colours, as there was no difference in neophobia or dietary conservatism towards either of the colours. Rowe and Guilford (1996) also noted that chicks treated yellow and red with the same level of innate avoidance; however, this is the first experiment to show the same levels of dietary conservatism towards red and yellow prey.
4.2 - EXPERIMENT 4 – A comparison of the effect of a unimodal, bimodal and trimodal signal on neophobia and dietary conservatism towards yellow crumbs.

4.2.1 – INTRODUCTION

There are many examples of aposematic insects utilising trimodal warning displays (Carpenter 1938; Rothschild 1961; Rothschild et al. 1984; Marples 1994). These consist of three component signals, such as the warning display of the Tiger moth (Artica caja) which comprises a visual, an olfactory and an auditory component (Rothschild 1961; Rothschild et al. 1984). Moore et al. (1990) noted that the bitter taste of many defended insects may also be regarded as a warning signal.

Marples et al. (1994) provide the only empirical investigation into the effect of a trimodal warning display on avian predator behaviour. Using quail (Coturnix coturnix japonica) as predators and seven-spotted ladybirds (Coccinella septempunctata) as prey, they examined a trimodal display comprised of the colourful elytra of the ladybird, the taste of its reflex blood, and the odour of a crushed ladybird. Colour was found to be the most important component cue, followed by taste, and then odour. However, they noted that the effect of the odour may have been reduced, as they used the odour of a crushed ladybird, which may have contained masking odours that reduced the effect of the pyrazine odour. Nevertheless, this study provides a good example that not all the component cues of a trimodal signal operate equally. They demonstrated that when all three component cues were present, avoidance approached that of the whole seven-spotted ladybird, which suggests that all three component signals interacted with one another to elicit predator avoidance.

Experiment 1 showed that pyrazine odour enhanced both neophobia and dietary conservatism of yellow prey, while Experiment 3 showed that buzzing did not. Rothschild et al. (1984) suggested that pyrazine odour may act as an alerting stimulus,
making predators more aware of the food they were eating; therefore, a logical progression was to investigate whether the presence of pyrazine odour would make the chicks more responsive to the buzzing sound. Experiment 4 sought to examine whether the addition of an auditory cue to the novel yellow, novel pyrazine signal further enhanced innate avoidance behaviour. This current experiment is the first to examine how a trimodal display operates free of an unpalatable taste.

4.2.2 - METHODS

The methods for this experiment were similar to those used in Experiment 2, except that buzzing was used as an additional signal in Treatment 2, with pyrazine odour in Treatment 3 and both buzzing and pyrazine odour in Treatment 4, and the aposematically coloured crumbs were yellow in all treatments (Table 4.2). All treatments had 13 replicates except for Treatment 4, which had 14.

Table 4.2: Treatments received during the training and test trials, indicated as crumb colours and odours. All crumbs were palatable.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Training food</th>
<th>Test food</th>
<th>Test for response to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green</td>
<td>Green; yellow</td>
<td>Novel yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green</td>
<td>Green; yellow plus buzzing</td>
<td>Novel yellow and novel buzzing</td>
</tr>
<tr>
<td>3</td>
<td>Green</td>
<td>Green; yellow plus pyrazine</td>
<td>Novel yellow and novel pyrazine</td>
</tr>
<tr>
<td>4</td>
<td>Green</td>
<td>Green; yellow plus pyrazine and buzzing</td>
<td>Novel yellow, novel pyrazine and novel buzzing</td>
</tr>
</tbody>
</table>
The olfactory and auditory signals had different effects on the neophobia that chicks exhibited towards the yellow crumbs. There were significant differences between the time to overcome neophobia of yellow crumbs by chicks between the four treatment groups (Kruskal-Wallis test, \( \chi^2 = 9.555, d.f. = 3, p < 0.05 \), Fig. 4.2.1). The chicks showed significantly greater neophobia towards yellow crumbs that smelt of pyrazine than towards odourless, silent yellow crumbs (Dunn’s post-hoc test Treatments 1 vs. 3 \( p < 0.05 \)), and towards the trimodal crumbs than the odourless, silent yellow crumbs (Dunn’s post-hoc test Treatments 1 vs. 4 \( p < 0.01 \)). Buzzing did not prolong neophobia of the yellow crumbs (Dunn’s post-hoc test Treatments 1 vs. 2 NS), and the addition of buzzing to the yellow pyrazine signal did not further prolong neophobia (Dunn’s post-hoc test Treatments 3 vs. 4 NS). There were no significant differences between the time to peck one green crumb by chicks in the four treatment groups (Kruskal-Wallis test, \( \chi^2 = 1.142, d.f. = 3, \text{NS} \), Fig. 4.2.1).

The chicks took longer to peck at familiar green crumbs than novel yellow crumbs in Treatment 1 (Mann-Whitney U test; \( U = 33.50, n = 13, 13, p < 0.01 \)). Chicks in all other treatments took longer to overcome neophobia of yellow crumbs than to peck at familiar green crumbs (Mann-Whitney U test; Treatment 2, \( U = 24.50, n = 13, 13, p < 0.001 \); Treatment 3, \( U = 23.00, n = 13, 13, p < 0.001 \); Treatment 4, \( U = 25.00, n = 14, 14, p < 0.001 \)). This suggests that once an additional signal was present the chicks were better able to differentiate between the familiar green crumbs and the novel yellow crumbs.
Figure 4.2.1: The mean latency (±s.e) (in seconds) to overcome neophobia of familiar green and novel yellow crumbs (indicated by the colour of the bars) with buzzing and/or pyrazine accompanying the yellow crumbs.

The pyrazine odour and buzzing sound also seemed to have different effects on the time taken to overcome dietary conservatism of the yellow crumbs (Kruskal-Wallis test, $\chi^2 = 19.005$, $d.f. = 3$, $p < 0.001$, Fig. 4.2.2). The addition of pyrazine odour prolonged dietary conservatism of yellow crumbs (Dunn’s post-hoc test Treatments 1 vs. 3 $p < 0.01$), whereas the addition of buzzing did not (Dunn’s post-hoc test Treatments 1 vs. 2 NS). The addition of buzzing to the yellow, pyrazine signal did not further prolong dietary conservatism towards the yellow crumbs (Dunn’s post-hoc test Treatments 3 vs. 4 NS). There were no significant differences between the time taken to overcome dietary conservatism of green crumbs by the four treatments (Kruskal-Wallis test, $\chi^2 = 1.163$, $d.f. = 3$, NS; Fig. 4.2.2).
The chicks did not differentiate between the yellow and green crumbs in Treatment 1, as there was also no significant difference in the times taken to overcome dietary conservatism of familiar green and novel yellow crumbs (Mann-Whitney U test; $U = 49.00$, $n = 13, 13$, NS). The addition of buzzing to the yellow crumbs in Treatment 2 did not change this (Mann-Whitney U test; $U = 48.50$, $n = 13, 13$, NS). When pyrazine was associated with the yellow crumbs, the chicks took significantly longer to overcome dietary conservatism of the yellow than the green crumbs (Mann-Whitney U test; Treatment 3, $U = 8.00$, $n = 13, 13$, $p < 0.001$; Treatment 4, $U = 20.50$, $n = 14, 14$, $p < 0.001$; Fig. 4.2.2).

![Bar chart showing mean latency (±s.e) to overcome dietary conservatism of familiar green and novel yellow crumbs with buzzing and/or pyrazine accompanying the yellow crumbs.](image)

**Figure 4.2.2**: The mean latency (±s.e) (in seconds) to overcome dietary conservatism of familiar green and novel yellow crumbs (indicated by the colour of the bars) with buzzing and/or pyrazine accompanying the yellow crumbs.

There were differences in the number of yellow crumbs eaten by chicks in each of the four treatments (Kruskal-Wallis test, $\chi^2 = 14.988$, $d.f. = 3$, $p < 0.01$, Fig. 4.2.3). The addition of a buzzing sound did not change the number of yellow crumbs eaten...
during the first trial (Dunn’s post-hoc test Treatment 1 vs. 2 NS). However, the addition of pyrazine odour decreased the number of yellow crumbs eaten (Dunn’s post-hoc test Treatment 1 vs. 3, p < 0.01). The addition of a buzzing sound to the yellow, pyrazine signal did not further decrease the number of yellow crumbs eaten (Dunn’s post-hoc test Treatment 3 vs. 4 NS). There were no significant differences in the number of green crumbs eaten across the four treatment groups (Kruskal-Wallis test, $\chi^2 = 6.835$, $d.f. = 3$, NS, Fig. 4.2.3).

**Figure 4.2.3:** The mean number (±s.e) of green and yellow crumbs (indicated by the colour of the bars) eaten during the first trial across the four treatment groups.

There were differences among the eating bias across the four treatment groups (Kruskal-Wallis test, $\chi^2 = 14.136$, $d.f. = 3$, p < 0.01, Fig. 4.2.4). The addition of buzzing to the yellow crumbs did not affect the eating bias (Dunn’s post-hoc test Treatment 1 vs. 2 NS). However, the addition of the pyrazine odour increased the
eating bias against yellow crumbs (Dunn’s post-hoc test Treatment 1 vs. 3, $p < 0.01$).

The addition of the buzzing to the yellow crumbs in the presence of pyrazine did not further increase the eating bias against yellow (Dunn’s post-hoc test Treatment 3 vs. 4 NS).

Figure 4.2.4: The eating bias ($\pm s.e$) (number of green minus number of yellow crumbs eaten) in the first trial across the four treatment groups.
4.2.4 – DISCUSSION

Pyrazine prolonged both neophobia and dietary conservatism towards yellow prey, which supports the findings of Experiment 1 and is in keeping with Rowe and Guilford’s results (1999a, b). Buzzing did not prolong either process of innate avoidance behaviour which is in keeping with Experiment 3. The addition of buzzing to the yellow pyrazine signal did not further prolong either neophobia or dietary conservatism, which suggests that with this particular combination of cues no additional protection was gained from signalling trimodally rather than signalling bimodally (using yellow and pyrazine). This experiment provided no evidence of pyrazine acting as an alerting stimulus to the buzzing signal, as the chicks did not show a heightened response to buzzing in the presence of the pyrazine odour (Rothschild et al. 1984). It may be that because a natural trimodal signal was not used that no additional effect was observed. The analysis of the number of crumbs of each colour eaten and the eating bias calculation both support the findings of the neophobia and dietary conservatism measures.

The chicks showed the same level of neophobia and dietary conservatism towards the yellow and green crumbs in Treatment 1; however, when an additional signal was present the chick discriminated between the green and yellow crumbs, as neophobia was longer towards the yellow than the green crumbs in all other treatments, and dietary conservatism was longer towards yellow than towards green in treatments where pyrazine was present.
These two experiments demonstrated that the agitated buzzing of *B. terrestris* played at 65-70 dB had no effect on either form of innate avoidance behaviour of chicks, and that the addition of the buzzing cue to the yellow crumbs that smelt of pyrazine signal did not further enhance either neophobia or dietary conservatism.

The results suggest that auditory signals may not have an effect on innate avoidance behaviour. There are many examples of insect warning sounds (Carpenter 1921; 1938; Blest 1957; Rothschild 1965; Bedford and Chinnick 1966; Masters 1979) and evidence of non-avian predators responding to these warning sounds (Myers 1935; Brower and Brower 1961; Kirchner and Roschard 1999). Given the good hearing capability of birds (Dooling 2002, 2004), it is difficult to understand why they would not attend to these auditory warnings. Experiments investigating the effects of other insect sounds may produce significant effects on innate avoidance behaviour and warrant future investigation.

Experiment 4 suggests that there is no additional protection to be gained from signalling trimodally rather than bimodally; however, this can only be said to be true for the signals tested in this experiment. There are many examples of natural trimodal warning displays (Rothschild 1984; Marples *et al.* 1994). Experiments such as those conducted here, could be used to determine the role of each component cue of these natural trimodal displays in eliciting predator avoidance.

The presence of buzzing appeared to help the chicks to discriminate between the red and green crumbs in Experiment 3; however, no such effect was observed with the yellow crumbs in either Experiments 3 or 4. Pyrazine, on the other hand, was observed to enhance discrimination between the yellow and green crumbs in
Experiments 1 and 4, but not between red and green crumbs (Experiment 2). This suggests that buzzing and pyrazine may interact with visual signals in different ways.

As noted in Chapter 3, the bright green used in Experiment 1 may have somewhat altered the chicks' responses to the novel crumbs; therefore, a paler green was used for all subsequent experiments. When the results from Experiments 1 and 4 were considered together, the effect of the paler green could be seen. The addition of the pyrazine to the yellow crumbs increased both neophobia and dietary conservatism of the yellow crumbs (Experiments 1 and 4). However, in Experiment 1 the pyrazine increased the neophobia and dietary conservatism towards the brighter green as well, but no such effect was seen when the paler green was used. In Experiment 1 it was noted that the addition of the pyrazine odour to the novel yellow crumbs did not alter the eating bias, but this was due to the effect of pyrazine on both the yellow and green crumbs. In contrast, in Experiment 4 the chicks showed a greater eating bias towards the yellow crumbs that smelt of pyrazine than towards the odourless yellow crumbs. When the data on number of crumbs eaten were examined, it was noted that the addition of pyrazine reduced the number of yellow crumbs eaten, but had no effect on the number of green crumbs eaten. These results suggest that the pyrazine interacted with the brighter green crumbs but had no effect on the paler, more familiar green crumbs used in all subsequent innate avoidance experiments.

If alternative, familiar, palatable food is available, then chicks are more likely to express wariness towards novel food items (Kelly and Marples 2004; Thomas et al. 2003; Lindström et al. 2000; Marples and Kelly 1999); therefore, the paler green used in Experiment 4 allowed the chicks to express a greater level of innate avoidance towards the yellow crumbs in the presence of pyrazine than the bright green crumbs in Experiment 1. It is interesting to note that the chicks still expressed innate avoidance
towards the yellow crumbs in the presence of the bright green crumbs, suggesting that the aversion to yellow is quite strong.

The four measures of innate avoidance - neophobia, dietary conservatism, number of crumbs eaten and eating bias - all gave similar results for Experiment 4; however, as noted in Chapter 3 and by Jetz et al. (2001), calculated measures may often fail to pick up on more subtle forms of innate avoidance behaviour, and therefore direct measures of latency offer a more sensitive measurement.
CHAPTER 5

The effect of pyrazine odour on learned avoidance and memory in chicks

Acknowledgement and authorship

The three experiments reported in this chapter were designed and analysed by E. Siddall; Dr. Marples contributed to the design of the experiments. The data for Experiments 5 and 6 of this chapter were collected by E. Siddall and the data for Experiment 7 by J. Beggs. J. Beggs was co-supervised by Dr. Brown and E. Siddall during her undergraduate moderatorship project for which these data were collected. Experiment 6 is published as “Better to be bimodal: the interaction of color and smell on learning and memory.” Siddall, E.C. & Marples, N. M. (2008) Behavioral Ecology, 19: 425 – 432.
As well as prolonging innate avoidance behaviour, once the predator has overcome its initial neophobia and started to sample the novel prey, pyrazines may enhance learning. Pyrazines can act as a discriminatory cue for learned avoidance in the absence of visual cues, as has been observed for both chicks (Guilford et al. 1987) and rats (Kaye et al. 1989). It has been suggested that the presence of pyrazine in a warning display may speed up the acquisition of learned avoidance (Rothschild and Moore 1987; Guilford and Dawkins 1991; Barnea et al. 2004; Section 1.3.2b) and the memorability of this avoidance (Rothschild 1984; Barnea et al. 2004). Barnea et al. (2004) provided some evidence of pyrazine odour enhancing learning and memory of unpalatable water. Two other non-warning odours, almond and vanilla have also been observed to enhance learned avoidance of distasteful water (Roper and Marples 1997a). However, Roper and Marples (1997b) suggested that birds may respond to solid and liquid prey differently. Pearce (1997) also suggested that the rates at which learning progresses with food and water may differ.

Once a predator has formed an association between the warning display and distastefulness of the prey, it must remember this association each time it meets the prey if the signal is to work as an effective defence (Guilford and Dawkins 1991; Speed 2000). Guilford et al. (1987) trained chicks to avoid colourless quinine-tainted water accompanied by pyrazine odour, then tested their memory of this association after a twenty-four hour retention interval. The chicks did not continue to avoid the water; however, they did show more signs of distress during the memory test than untrained chicks, suggesting some recollection of the meaning of the pyrazine odour. This study was conducted without a colour cue, and therefore represents the memorability of pyrazine as a lone cue.
If defended insects which signal multimodally are better protected, and remembered for longer, then it is to be expected that mimics of multimodal model species will be better protected than mimics of monomodal species. There are many examples of multimodal mimicry by insect species (Moore et al. 1990) but until the present study, no research has investigated the difference in protection gained by mimicking a monomodal or a multimodal model species. It is unknown whether mimics of multimodal models need to mimic all components of the multimodal signal, or whether mimicry of the visual component alone is sufficient to give full protection.

The following experiments investigate whether pyrazine odour enhances learned avoidance of unpalatable warningly coloured prey, and how well this learned avoidance is remembered after a retention interval. The avoidance of mimics of the unpalatable prey is also examined. Finally, the effect of the pyrazine odour on generalisation of the learned avoidance of the defended crumbs to palatable crumbs in close proximity is investigated. Such generalisation based on a secondary cue, such as odour, has not been considered in previous studies.

5.2 EXPERIMENT 5 – The effect of pyrazine odour on learned avoidance and memory of bright yellow crumbs.

5.2.1 – METHODS

The experiment was conducted over two weeks with 7 chicks in each treatment per week to give a final replicate number of 14 chicks per treatment. Mann-Whitney U tests showed that there was no significant difference between the data from the two weeks, and therefore the data were combined and analysed. The pre-training and familiarisation training on days one and two were the same as in Experiment 1 (Section
3.1.2) On day three the chicks were offered a three-minute learning trial, with a choice test between twelve palatable green crumbs and twelve unpalatable yellow crumbs in the presence or absence of pyrazine odour (Table 5.1). During these test sessions the number of crumbs of each colour eaten was noted. The chicks were given a total of six such learning trials, three on each of days three and four. Ninety-six hours after the last learning trial, the chicks were given a three-minute extinction trial in which all crumbs were palatable (Table 5.1), and again the number of crumbs of each colour eaten was noted. The yellow used in this experiment was the same as the yellow used in the learned avoidance experiments (Section 2.2.1).

Table 5.1: Treatments received during the learning and extinction trials, indicated as crumb colour and odour. Yellow crumbs were unpalatable in the learning trials. All crumbs were palatable in the extinction trials.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>familiar food</th>
<th>learning trials</th>
<th>extinction trials (96 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green</td>
<td>Green; yellow</td>
<td>Green; yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green</td>
<td>Green; yellow with pyrazine</td>
<td>Green; yellow with pyrazine</td>
</tr>
</tbody>
</table>

96
5.2.3 – RESULTS

In the first learning trial chicks ate significantly fewer yellow crumbs when the pyrazine odour was present (Mann-Whitney U test, $U = 51.00, n = 14, 14, p < 0.05$, Fig. 5.2.1), which suggests that pyrazine odour enhanced innate avoidance of yellow crumbs. After the first learning trial, there was no significant difference between the number of yellow crumbs eaten by the two treatment groups. Chicks in the odourless treatment learned to avoid the unpalatable yellow crumbs by the last learning trial as they ate significantly fewer crumbs in the last learning trial, than in the first learning trial (Mann-Whitney U test, $U = 35.00, n = 14, 14, p < 0.01$). Chicks in the pyrazine treatment showed no difference between the number of yellow crumbs attacked during the first and last learning trials (Mann-Whitney U test, $U = 90.00, n = 14, 14, NS$). However, chicks in both the pyrazine and odourless treatments ate the same number of yellow crumbs in the last learning trial (Mann-Whitney U test, $U = 91.00, n = 14, 14, NS$). This suggests that in the presence of pyrazine, the chicks showed such a high level of innate avoidance that they may not have encountered the yellow crumbs enough to learn to avoid them. It also may be that the chicks learned to avoid the yellow crumbs during the first learning trial, and therefore could not learn any further avoidance during the subsequent learning trials.
Figure 5.2.1: The mean number (±s.e) of yellow crumbs eaten across the six learning trials by chicks in the odourless (solid line) and pyrazine (dashed line) treatments, N = 14 for both treatments.

Even though after the first learning trial there was no significant difference between the two treatments, chicks in the odourless treatment ate a greater total number of yellow crumbs than chicks in the pyrazine treatment (Mann-Whitney U test, $U = 49.50$, $n = 14, 14$, $p < 0.05$, Fig. 5.2.2), so crumbs that smelt of pyrazine were avoided more than odourless crumbs.
There was no significant difference in the number of green crumbs eaten by the chicks in the odourless treatment across the six learning trials (Kruskal-Wallis test, $\chi^2 = 4.909$, $d.f. = 5$, NS, Fig. 5.2.3). Chicks in the odourless treatment also ate the same number of yellow and green crumbs during each of the six learning trials, suggesting that the chicks treated the palatable green crumbs the same way as the unpalatable yellow crumbs.
Figure 5.2.3: The mean number (± s.e) of green (squares) and yellow (triangles) crumbs eaten by chicks in the odourless treatment across the six learning trials.

In contrast, the number of green crumbs eaten by chicks in the pyrazine treatment differed significantly across the six learning trials (Kruskal-Wallis test, $\chi^2 = 13.561, d.f. = 5, p < 0.05$, Fig. 5.2.4). The chicks ate significantly more green than yellow crumbs in the first learning trial (Mann-Whitney U test, $U = 41.50, n = 14, 14, p < 0.01$), which supports the findings of Experiment 1 in which the pyrazine odour enhanced innate avoidance of yellow crumbs. However, in the subsequent trials there were no significant differences between the number of yellow and green crumbs eaten by the chicks in each trial.
Figure 5.2.4: The mean number (±s.e) of green (squares) and yellow (triangles) crumbs eaten by chicks in the pyrazine treatment across the six learning trials.

Chicks in the odourless treatment showed significant differences in the number of yellow crumbs they ate across the first and last learning trial, and the extinction test (Kruskal-Wallis test, $\chi^2 = 15.528$, $d.f. = 2$, $p < 0.001$, Fig. 5.2.5). Chicks learned to avoid the yellow crumbs by the last learning trial, as they ate significantly fewer crumbs in the last learning trial than the first learning trial (Dunn’s post-hoc test trial 1 vs. 6 $p < 0.01$). They remembered this learned avoidance after the 96-hour retention interval, as they ate fewer crumbs in the extinction trial than the first learning trial (Dunn’s post-hoc test trial 1 vs. 7 $p < 0.05$), and there was no significant difference in the number of yellow crumbs eaten between the last learning trial and the 96-hour extinction trial (Dunn’s post-hoc test trial 6 vs. 7 NS). However, chicks in the pyrazine treatment showed no significant difference in the number of yellow crumbs eaten across the three trials (Kruskal-Wallis test, $\chi^2 = 0.583$, $d.f. = 2$, NS).
5.2.4 – DISCUSSION

This experiment supports the findings of Experiment 1, as it shows that pyrazine odour enhanced innate avoidance of yellow prey, as also reported by Rowe and Guilford (1999b). The chicks in the pyrazine treatment seemed to learn an avoidance of the yellow crumbs during the first learning trial, therefore giving the non-significant values observed. Chicks in both treatments avoided the yellow crumbs to the same level by the last learning trial. When the total number of yellow crumbs eaten across the learning trials was examined, it was noted that chicks in the pyrazine treatment ate significantly fewer yellow crumbs than chicks in the odourless treatment, suggesting that pyrazine enhances the learned avoidance of yellow crumbs. Lindström
(1999) suggests that a predator may acquire a learned avoidance of a warning signal faster if it already expresses an innate avoidance of the signal.

In the first learning trial, chicks in the odourless treatment ate the same number of green and yellow crumbs, whereas chicks in the pyrazine treatment ate fewer yellow than green crumbs, which suggests that the pyrazine odour helped the chicks to differentiate between the green and yellow crumbs. However, by the second learning trial chicks in the pyrazine treatment ate the same number of green and yellow crumbs, suggesting the learned avoidance of the unpalatable yellow crumbs was generalised to the palatable green crumbs.

It would appear from these results that pyrazine odour had no effect on memory; however, as neither treatment forgot their learned avoidance after the 96-hour retention interval this cannot be determined conclusively. Rescorla and Wagner (1972) noted that the intensity of the conditioned stimuli (the yellow and pyrazine cues) and the unconditioned stimulus (the unpalatable flavour of bitrex) affect the rate of learning. It was therefore felt that using a paler yellow might slow the rate of avoidance learning of the yellow, pyrazine signal, allowing any effects of the pyrazine odour on learning and memory to be observed more clearly. Also, as discussed in Section 3.3, the brightness of the green used in this experiment may have altered the chicks' responses to the yellow crumbs, therefore a paler green was used in all subsequent learning experiments.

The concentration of bitrex used in this experiment was also very strong (see Section 2.2.2), and therefore learning may have progressed very rapidly. Reducing the intensity of the bitrex concentration may slow the rate of learning, thus making it more observable, and may allow differences in the rates to be detected. A second experiment was therefore designed in which the yellow and green were less intense and the bitrex concentration lower, and is reported here as Experiment 6.
5.3 - EXPERIMENT 6 – The effect of pyrazine odour on learned avoidance and memory of pale yellow crumbs.

5.3.1 – INTRODUCTION

This experiment sought to further investigate whether pyrazine odour enhanced learned avoidance of unpalatable yellow crumbs, and the memorability of this learned avoidance. In addition to the colour changes just discussed in Section 5.2.4, in this experiment an additional learning trial was added to the experimental design to follow the methodology used by Skelhorn and Rowe (2005). Also, the number of crumbs attacked rather than the number of crumbs eaten was recorded, as it was felt that it would better reflect the results from all learning events, including those where the chick did not swallow the crumb. In addition to the 96-hour extinction trial carried out on one subset of chicks, another extinction trial was conducted on another subset of chicks 3 hours after the last learning trial. The aim of this 3-hour extinction trial as discussed in Section 2.6.2 was to test whether learning had indeed occurred over the course of the seven learning trials. It offered the chicks the yellow conditioned stimulus in the absence of the bitrex unconditioned stimulus. If the chick avoided the yellow crumbs during this trial, it would confirm that they had acquired a learned avoidance of the yellow crumbs. The 96-hour extinction trial then served to determine how well the chicks remembered their learned avoidance after a longer retention interval.
5.3.2 – METHODS

The experiment was conducted over two weeks, with seven chicks in each treatment in the first week and six in each treatment in the second week, to give a final replicate number of 12 or 13 chicks per treatment. Mann-Whitney U tests showed that there were no significant differences between the data from the two weeks, and therefore the data were combined and analysed. The pre-training was conducted on day one, as described in Section 2.4. On day two the chicks were offered a three-minute learning trial with a choice test between twelve palatable green crumbs and twelve unpalatable red crumbs in the presence or absence of pyrazine (Table 5.2). During these learning trials the number of each colour of crumb attacked was noted. The chicks were given a total of seven such learning trials, three on each of days two and three and one learning trial on day four. The chicks in each treatment were then subdivided into two extinction groups, one of which completed an extinction trial three hours after the last learning trial, and the other of which completed its extinction trial 96 hours after the last learning trial (Table 5.2). During these extinction trials all crumbs were palatable, and again the number of each colour of crumb attacked was noted.

Table 5.2: Treatments received during the learning and extinction trials, indicated as crumb colour and odour. Yellow crumbs were unpalatable during the learning trials. All crumbs were palatable during the extinction trials.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>learning trials</th>
<th>extinction trials (3hours or 96hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green; yellow</td>
<td>Green; yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green; yellow</td>
<td>Green; yellow with pyrazine</td>
</tr>
<tr>
<td>3</td>
<td>Green; yellow with pyrazine</td>
<td>Green; yellow</td>
</tr>
<tr>
<td>4</td>
<td>Green; yellow with pyrazine</td>
<td>Green; yellow with pyrazine</td>
</tr>
</tbody>
</table>
Chicks in both the pyrazine and odourless treatments learned to avoid the unpalatable yellow crumbs over the course of the seven learning trials, attacking significantly fewer crumbs during trial seven than during trial one (Mann-Whitney U-test, odourless treatments: \( U = 846.00; n = 50, 50; p < 0.01 \) and pyrazine treatments: \( U = 424.00; n = 50, 50; p < 0.001 \); Fig. 5.3.1 and 5.3.4).

In the first trial, there was no significant difference in the number of yellow crumbs attacked in the presence or absence of the pyrazine odour (Mann-Whitney U-test, \( U = 1230.50; n = 50, 50; \text{NS} \); Fig. 5.3.1 and 5.3.4). Therefore, contrary to previous findings (Rowe and Guilford 1996, 1999a, and Experiment 1 and 5 of this thesis), pyrazine did not increase the innate avoidance of novel yellow crumbs in this instance, possibly because a pale yellow was used in this experiment. By trial three, chicks in the pyrazine treatment were attacking significantly fewer yellow crumbs than the chicks in the odourless treatment (Mann-Whitney U-test, \( U = 987.00; n = 50, 50; p < 0.05 \); Fig. 5.3.1). This difference was maintained during all subsequent learning trials, indicating faster acquisition of the learned avoidance in the presence of pyrazine.

In the last learning trial chicks in the pyrazine treatment attacked significantly fewer yellow crumbs than chicks in the odourless treatment (Mann-Whitney U-test, \( U = 826.50; n = 50, 50; p < 0.01 \); Fig. 5.3.1). This indicates that after the seven learning trials, the chicks learned to avoid the yellow odorous crumbs to a greater extent than the yellow odourless crumbs.
Figure 5.3.1: The mean number (±s.e) of yellow (triangles) and green (squares) crumbs attacked across the seven learning trials by chicks in the odourless (solid line) and pyrazine (dashed line) treatments.

Chicks in the pyrazine treatment attacked significantly fewer yellow crumbs across all the learning trials than chicks in the odourless treatment (Mann-Whitney U-test, U = 941.50, n = 50, 50, p < 0.05; Fig. 5.3.2). These results suggest that prey that is both yellow and smells of pyrazine is better protected than prey that is just yellow alone.
The chicks were offered palatable green crumbs as an alternative food to the yellow crumbs throughout the experiment. There were no significant differences between the number of green and yellow crumbs attacked by chicks in either the odourless or the pyrazine treatment during any of the learning trials (Fig. 5.3.1). This suggests that the chicks treated the green and yellow crumbs in the same manner throughout the experiment. However, when the relative proportion of each crumb colour attacked was examined, the chicks in the pyrazine treatment appeared to differentiate between the green and yellow crumbs, whereas chicks in the odourless treatment did not (Fig. 5.3.3). As they acquired the learned avoidance, chicks in all treatments reduced the number of yellow crumbs they attacked, but their response to the green crumbs differed. In the odourless treatment, the chicks reduced the number of green as well as yellow crumbs attacked, so the relative number of yellow crumbs attacked did not change across the learning trials (Kruskal-Wallis test, $\chi^2 = 4.203$; d.f. = 6; $p = \text{NS}$; Fig. 5.3.3). In contrast, the chicks in the pyrazine treatment differentiated between the colours, continuing to attack the green crumbs while they learned to avoid
the yellow crumbs, so the relative proportion of yellow crumbs attacked reduced across the learning trials (Kruskal-Wallis test, $\chi^2 = 39.530$; d.f. = 6; $p < 0.001$; Fig. 5.3.3). After four learning trials, the chicks in the pyrazine treatment had learned to avoid the unpalatable yellow crumbs and to distinguish between the colours (Fig. 5.3.3; for post hoc tests of these comparisons see Table 5.3). This result suggests that pyrazine odour reduced the chicks’ tendency to generalise between the colours and made them more able to discriminate against the unpalatable yellow crumbs, eating proportionately more palatable green crumbs.

![Figure 5.3.3: The mean proportion (±s.e) of yellow crumbs attacked across the seven learning trials relative to the number of green crumbs attacked by the odourless (solid line) and pyrazine (dashed line) treatments. N = 25 per treatment.](image)
Table 5.3: Dunn’s post-hoc test results for the relative number of yellow crumbs attacked by chicks in the pyrazine treatment across the seven learning trials. All comparisons for the odourless treatments were NS.

<table>
<thead>
<tr>
<th>Training trial</th>
<th>rank difference</th>
<th>critical value</th>
<th>p-value &lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 v 5</td>
<td>76.1</td>
<td>68.58</td>
<td>0.001</td>
</tr>
<tr>
<td>1 v 6</td>
<td>92.28</td>
<td>68.58</td>
<td>0.001</td>
</tr>
<tr>
<td>1 v 7</td>
<td>74.56</td>
<td>68.58</td>
<td>0.001</td>
</tr>
<tr>
<td>2 v 5</td>
<td>52.00</td>
<td>52.08</td>
<td>0.05</td>
</tr>
<tr>
<td>2 v 6</td>
<td>68.18</td>
<td>39.63</td>
<td>0.01</td>
</tr>
<tr>
<td>2 v 7</td>
<td>50.46</td>
<td>39.63</td>
<td>0.05</td>
</tr>
<tr>
<td>3 v 5</td>
<td>44.26</td>
<td>39.63</td>
<td>0.05</td>
</tr>
<tr>
<td>3 v 6</td>
<td>60.44</td>
<td>52.08</td>
<td>0.01</td>
</tr>
<tr>
<td>3 v 7</td>
<td>42.72</td>
<td>39.63</td>
<td>0.05</td>
</tr>
<tr>
<td>4 v 5</td>
<td>60.01</td>
<td>52.08</td>
<td>0.01</td>
</tr>
<tr>
<td>4 v 6</td>
<td>76.19</td>
<td>68.56</td>
<td>0.001</td>
</tr>
<tr>
<td>4 v 7</td>
<td>58.47</td>
<td>52.08</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The chicks in all treatments attacked significantly different numbers of yellow crumbs during the first and last learning trials and the two extinction trials (Kruskal-Wallis test, Treatment 1 $\chi^2 = 15.494$; d.f. = 3; p < 0.01; Treatment 2 $\chi^2 = 11.672$; d.f. = 3; p < 0.01; Treatment 3 $\chi^2 = 41.901$; d.f. = 3; p < 0.001; Treatment 4 $\chi^2 = 46.064$; d.f. = 3; p < 0.001; Fig. 5.3.4).

As already stated, the chicks in the pyrazine treatments (Treatments 3 and 4) learned to avoid the yellow crumbs to a greater extent than the chicks in the odourless treatments (Treatment 1 and 2). This difference in avoidance levels between the four treatments was no longer detectable after the 3-hour consolidation period (Kruskal-Wallis test, $\chi^2 = 1.439$; d.f. = 3; NS; Fig. 5.3.4). Chicks in the odourless treatments (Treatments 1 and 2) attacked significantly fewer yellow crumbs during the 3-hour extinction trial than they did during their final learning trial (Dunn’s post-hoc test for Treatment 1: trial 7 vs. 8, p < 0.001; Treatment 2: trial 7 vs. 8, p < 0.001; Fig. 5.3.4). There was no such difference shown by chicks in the pyrazine treatments (Treatments 3
and 4) during these two trials (Dunn’s post-hoc test for Treatment 3: trial 7 vs. 8, NS; Treatment 4: trial 7 vs. 8, NS; Fig. 5.3.4).

After a 96-hour retention interval, chicks in Treatments 1, 2 and 3 showed signs of forgetting their learned avoidance, whereas chicks in Treatment 4 did not (Fig. 5.3.4). There was a significant difference in the level of avoidance shown by the treatments after the 96-hour retention interval (Kruskal-Wallis test across all four treatments, $\chi^2 = 9.555$; d.f. = 3; $p < 0.05$; Fig. 5.3.4). There was no significant difference in the number of yellow crumbs attacked by chicks in Treatments 1 and 2; therefore, the addition of pyrazine to prey during the extinction trial in Treatment 2 did not enhance recollection of the learned avoidance.

Chicks in Treatment 4 attacked significantly fewer yellow crumbs after the 96-hour retention interval than chicks in Treatments 1 and 2 (Dunn’s post-hoc test Treatment 1 vs. 4; $p < 0.001$; Treatment 2 vs. 4; $p < 0.001$; Fig. 5.3.4). From Fig. 5.3.4 it appears that chicks in Treatment 3 forgot their learned avoidance to a level intermediate between Treatments 1 and 2 and Treatment 4, showing no significant difference to any other treatment (Dunn’s post-hoc test Trial 1 vs. 3; NS; Trial 2 vs. 3; NS; Trial 3 vs. 4; NS). Although not significant, these results suggest that chicks that learned to avoid yellow, pyrazine crumbs but were presented with yellow, odourless crumbs avoided them to an intermediate level between chicks that were offered yellow, pyrazine crumbs in both the learning and memory trials, and chicks that learned to avoid the odourless yellow crumbs.

Chicks in Treatments 1, 2 and 3 attacked significantly more crumbs during the 96-hour extinction trial than their counterparts did during the 3-hour extinction trial (Dunn’s post-hoc test Treatment 1, trial 8 vs. 9, $p < 0.001$; Treatment 2, trial 8 vs. 9, $p < 0.001$; Treatment 3, trial 8 vs. 9, $p < 0.001$), suggesting that they forgot their learned avoidance to some extent. However, they attacked significantly fewer crumbs than
they did during the first learning trial (Dunn’s post-hoc test Treatment 1, trial 1 vs. 9, \( p < 0.001 \); Treatment 2, trial 1 vs. 9, \( p < 0.001 \); Treatment 3, trial 7 vs. 9, \( p < 0.001 \)), suggesting that they had not forgotten their avoidance completely.

Chicks in Treatment 4 showed no evidence of forgetting after the 96-hour retention interval. There were no significant differences in the number of yellow crumbs attacked during the last learning trial, the 3-hour and 96-hour extinction trials (Kruskal-Wallis test, \( \chi^2 = 1.893 \); d.f. = 2; \( p = \text{NS} \); Fig. 5.2.4). Thus, the yellow, pyrazine crumbs were as well protected after the 96-hour interval as they were after the 3-hour consolidation period.

**Figure 5.3.4:** The mean number (±s.e) of yellow crumbs attacked in the first and last learning trials, and the 3-hour and 96-hour extinction trials.
This experiment clearly demonstrates that the addition of pyrazine odour to a yellow visual signal increased the rate and degree of avoidance learning of solid prey and prolonged memorability of the learned avoidance in chicks. Barnea et al. (2004) observed a similar effect of pyrazine with red but failed to see an increased learning rate with yellow.

During the final learning trial the chicks in the odourless treatment attacked significantly more yellow crumbs than the chicks in the pyrazine treatment. However, after the 3-hour retention interval this difference disappeared. These results support Hale and Crowe’s (2002) view that memory consolidation proceeds for several hours following learning. It also suggests that the presence of pyrazine during learning made learning so effective that the consolidation had already happened by the seventh learning trial, so birds in Treatments 3 and 4 did not improve their avoidance during the consolidation period. Consolidation of the learned avoidance by Treatments 1 and 2 may therefore have caused the difference in attack levels between all treatments to disappear.

After the 96-hour retention interval the chicks remembered a learned avoidance of the yellow crumbs that smelt of pyrazine, but forgot the learned avoidance of the odourless yellow crumbs. This confirms Barnea et al.’s (2004) result that pyrazine odour prolongs memory of learned avoidance in a similar manner to other odours such as almond (Roper and Marples 1997a). These results are in keeping with Roper and Redston’s (1987) suggestion that conspicuousness prolongs memory, assuming that smell enhances conspicuousness. The chicks in the pyrazine treatment attacked fewer yellow crumbs than the chicks in the odourless treatment; therefore, the observed increases in learning and memory are presumably due to some aspect of the signal that
makes it memorable rather than an enhanced encounter rate, as debated by Gittleman and Harvey (1980) and Roper and Redston (1987). Also, as the chicks in all treatments consolidated their learning to the same level by the 3-hour extinction trial, the prolonged memory in Treatment 4 appears to be due to something inherently memorable about the signal rather than to a greater degree of learning. The results also suggest that even after 96 hours, mimics of a monomodal model (Treatments 1 and 2) and inaccurate mimics of the multimodal model (Treatment 3) are better protected if the predator is educated rather than naïve.

When the proportion of each colour of crumb attacked during the learning trials was examined, it became clear that the presence of pyrazine improved discrimination between the palatable green and the unpalatable yellow crumbs. In the odourless treatment, the chicks generalised their learned avoidance of yellow to include the green crumbs. This is similar to the Mappes et al. (1999) observation that palatable species living in proximity to unpalatable species may gain protection due to avoidance of all prey in the patch by the predator. The generalisation between yellow and green may have hindered learning in the odourless treatments to some extent, but it did not prevent it, as the chicks gradually avoided both colours of crumbs. This suggests that they were learning avoidance, but that this avoidance was of both colours rather than just towards the colour of the defended prey.

In the presence of pyrazine, the generalisation of the learned avoidance of yellow prey to the palatable green prey was reduced, and the birds were better able to discriminate between the two colours. This reduced generalisation suggests that although the pyrazine odour was pervasive throughout the entire experimental arena, the chicks could detect it more strongly beneath the wells that contained yellow crumbs and used this relative odour strength further to distinguish between the two crumb colours. Kraemer (1984) noted that memorability may be enhanced if the two signals
in a discrimination process are distinct from one another. The addition of the pyrazine to the yellow crumbs in this experiment appears to have enhanced their distinctiveness which may have contributed to the signal's memorability.

An alternative explanation for this difference in colour discrimination is that the pyrazine odour may have acted as an alerting signal making the chicks pay more attention to the aposematically coloured yellow crumbs than the green crumbs (Rothschild and Moore 1987). These results may therefore support the suggestion that pyrazine has an alerting function making the predator more aware of the features of the prey it is eating.

Memorability of a learned avoidance has implications for the success of mimetic species (Speed 2001). If the chicks' responses to the palatable yellow crumbs in the 3-hour and 96-hour extinction tests are considered comparable to wild birds' responses to Batesian mimics, then the results support this assertion, (for discussion see Section 1.6). After the 3-hour retention interval, all chicks had consolidated their learned avoidance to the same level, which suggests that there may be no difference in the protection of multimodal and monomodal mimics (if they were encountered by the predator after a period of consolidation following an encounter with the distasteful model, but before forgetting of the association had commenced). This would suggest that mimetic insects living sympatrically with a multimodal model would gain no extra protection from mimicking both the visual and the olfactory components of the display, since the visual component provides sufficient protection.

After a 96-hour retention interval the mimics in all treatments were better protected than they would have been through innate avoidance alone; however, mimics of the multimodal model were better protected than mimics of the monomodal model. The addition of pyrazine to a yellow mimic of an odourless model did not enhance avoidance, which suggests that in this instance pyrazine did not operate as an alerting
signal as suggested by Rothschild et al. (1984). However as the learned avoidance had been forgotten in Treatment 1, it may be that the addition of the pyrazine odour could not jog the chicks memory as the learned avoidance had already dissipated.

Crumbs that mimicked only the visual component of the multimodal signal appeared to be protected to an intermediate level between crumbs that mimicked both components of the multimodal signal and mimics of the monomodal crumbs. However, Rothschild (1984) suggests that mimics do not need to replicate their model’s defences perfectly and that a “reminder of the danger involved is sufficient”. Further work is needed to determine whether a mimic needs to replicate both components cues of a multimodal display in order to gain full protection.
5.4 - EXPERIMENT 7 – The effect of pyrazine odour on learned avoidance and memory of red crumbs.

5.4.1 – INTRODUCTION

This experiment sought to investigate further whether pyrazine odour enhanced learned avoidance of unpalatable red crumbs and the memorability of this learned avoidance in a similar manner to that observed with yellow crumbs in Experiment 6.

5.4.2 - METHODS

The method was similar to that used in Experiment 6, but with red crumbs used in place of yellow crumbs (Table 5.4). The experiment was conducted over four weeks with 4 chicks in each treatment per week to give a final replicate number of 16 chicks per treatment. Mann-Whitney U tests showed no significant difference between the data across weeks; therefore, the data were combined for analysis.

Table 5.4: Treatments received during the learning and extinction trials, indicated as crumb colour and odour. Red crumbs were unpalatable during the learning trials. All crumbs were palatable during the extinction trials.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>learning trials</th>
<th>extinction trials (3 hours or 96 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green; red</td>
<td>Green; red</td>
</tr>
<tr>
<td>2</td>
<td>Green; red</td>
<td>Green; red with pyrazine</td>
</tr>
<tr>
<td>3</td>
<td>Green; red with pyrazine</td>
<td>Green; red</td>
</tr>
<tr>
<td>4</td>
<td>Green; red with pyrazine</td>
<td>Green; red with pyrazine</td>
</tr>
</tbody>
</table>
5.4.3 – RESULTS

All chicks learned to avoid the red crumbs by the last learning trial, indicated by a significant difference between the number of red crumbs attacked during the first and last learning trials, for both the odourless treatment (Mann-Whitney U test, \( U = 1112.00, n = 64, 64, p < 0.001, \) Fig. 5.4.1), and the pyrazine treatment (Mann-Whitney U test, \( U = 1372.50, n = 64, 64, p < 0.001, \) Fig. 5.4.1). During the first learning trial, chicks in the odourless treatment attacked significantly more red crumbs than chicks in the pyrazine treatment (Mann-Whitney U test, \( U = 1610.50, n = 64, 64, p < 0.05),\) supporting the earlier findings of greater innate aversion in the presence of pyrazine than in its absence. However, this difference disappeared by trial two and was not seen for the rest of the learning trials. The chicks learned to avoid the red crumbs to the same level regardless of whether they smelled of pyrazine (Mann-Whitney U test, Trial 7; \( U = 1893.50, n = 64, 64, \) NS).

In the first learning trial chicks in the odourless treatment attacked the same number of red and green crumbs (Mann-Whitney U test, \( U = 1964.50, n = 64, 64, \) NS). This was also the case for chicks in the pyrazine treatment (Mann-Whitney U test, \( U = 1939.00, n = 64, 64, \) NS, Fig. 5.4.1). However, by the second learning trial chicks in both treatments attacked significantly fewer red than green crumbs (Mann-Whitney U test, odourless \( U = 1587.50, n = 64, 64, p < 0.05);\) pyrazine \( U = 1532.50, n = 64, 64, p < 0.01)\) and this difference remained throughout all subsequent learning trials.
Figure 5.4.1: The mean number (±s.e) of red (triangles) and green (squares) crumbs attacked across the seven learning trials by chicks in the odourless (solid line) and pyrazine (dashed line) treatments.

Chicks in the odourless treatment attacked significantly more red crumbs during the course of the seven learning trials than chicks in the pyrazine treatment (Mann-Whitney U test, $U = 1537.00$, $n = 64, 64$, $p < 0.05$, Fig. 5.4.2), which suggests that crumbs that were red and smelt of pyrazine were better protected than crumbs that were just red, although this comparison does not differentiate between whether this protection is afforded through innate avoidance or enhanced learning. Skelhorn and Rowe (2006) used a similar measure of learning and concluded that a significant decrease in the number of crumbs attacked across the learning trials indicated a faster acquisition of avoidance learning.
Figure 5.4.2: The mean of the total number (±s.e) of crumbs attacked across the seven learning trials by the odourless and pyrazine treatments.

The chicks in the odourless and pyrazine treatments avoided the red crumbs to the same level during the 3-hour extinction trial (Kruskal-Wallis test, $\chi^2 = 3.552$, d.f. = 3, NS, Fig. 5.4.3). They also remembered this learned avoidance to the same extent after the 96-hour retention interval regardless of whether the crumbs smelt of pyrazine or were odourless (Kruskal-Wallis test, $\chi^2 = 1.915$, d.f. = 3, NS, Fig. 5.4.3), suggesting that the presence of the pyrazine odour made no difference to the memorability of the red signal.

Chicks in all treatments attacked significantly different numbers of red crumbs during the first and last learning trials and the two extinction trials (Kruskal-Wallis test, Treatment 1 $\chi^2 = 26.735$, d.f. = 3, p < 0.001; Treatment 2 $\chi^2 = 33.653$, d.f. = 3, p < 0.001; Treatment 3 $\chi^2 = 15.387$, d.f. = 3, p < 0.01; Treatment 4 $\chi^2 = 18.520$, d.f. = 3, p < 0.001; Fig. 5.4.3), suggesting that all chicks learned avoidance of the red crumbs over the course of the experiment.
Chicks in Treatment 1 that learned to avoid odourless red crumbs and were then offered odourless red crumbs in the extinction trials continued to avoid the crumbs after both the 3-hour (Dunn's post-hoc test trial 1 vs. 8 p < 0.01) and 96-hour retention intervals (Dunn's post-hoc test trial 1 vs. 9 p < 0.05). The chicks in Treatment 2 also avoided the red crumbs when they smelt of pyrazine during the 3-hour (Dunn's post-hoc test trial 1 vs. 8 p < 0.001) and 96-hour extinction trials (Dunn's post-hoc test trial 1 vs. 9 p < 0.01). Thus, chicks that learned without an odour component remembered their avoidance of red even after the 96-hour retention interval.

It appears that chicks that learned to avoid the red crumbs that smelt of pyrazine needed both component cues of the multimodal signal to be present in order for the avoidance to be fully remembered. In Treatment 3 chicks acquired a learned avoidance of red crumbs that smelt of pyrazine, but were offered odourless red crumbs during the extinction trials. There was no significant difference between the number of red crumbs attacked in the first learning trial and the 3-hour (Dunn's post-hoc test trial 1 vs. 8 NS) or 96-hour extinction trials (Dunn's post-hoc test trial 1 vs. 9 NS), suggesting that both the pyrazine and red signals needed to be present in order for the chicks to remember their learned avoidance. However, when chicks in Treatment 4 were offered red crumbs that smelt of pyrazine during the extinction trials; they remembered their learned avoidance after both the 3-hour (Dunn's post-hoc test trial 1 vs. 8 p < 0.01) and 96-hour retention intervals (Dunn's post-hoc test trial 1 vs. 9 p < 0.05).
Figure 5.4.3: The mean number of (±s.e) red crumbs attacked in the first and last learning trials, and the 3-hour and 96-hour extinction trials.

5.4.4 – DISCUSSION

During the first learning trial the chicks attacked fewer red crumbs that smelt of pyrazine than odourless red crumbs. This may be because the chicks expressed a higher level of innate avoidance towards the red crumbs that smelt of pyrazine than towards red crumbs alone, which supports the findings of Experiment 2. Alternatively, the pyrazine odour may have increased the rate of learning in the first trial.

Because learned aversion towards red prey was acquired so quickly, no difference in the rate of avoidance learning in the presence or absence of the pyrazine odour could be detected. However, the presence of pyrazine reduced the total number
of red crumbs attacked across all the learning trials, suggesting that pyrazine did enhance learned avoidance of red crumbs.

Pyrazine did not affect how well the birds remembered their learned avoidance, but this may be because the birds remembered the red visual signal so well that the pyrazine odour had little effect. The chicks that learned to avoid odourless red crumbs remembered this avoidance after 96 hours as well as chicks that learned to avoid red crumbs that smelt of pyrazine; therefore, the red visual signal may have overshadowed any effect of pyrazine on memorability. There is some evidence that if the chicks learned to avoid crumbs that smelled of pyrazine, the odour needed to be present during the extinction trials in order for them to remember their learned avoidance. This suggests that a mimic of a model with a red, pyrazine warning display would be better protected if it mimicked both components of the warning display rather than just the visual cue.

After the first learning trial, the chicks did not appear to generalise between the red and green crumbs, as they continued to attack the palatable green crumbs while learning to avoid the unpalatable red crumbs. The addition of pyrazine odour did not alter this.
5.5 - GENERAL DISCUSSION

The experiments from this chapter confirm that pyrazine odour enhances learned avoidance of unpalatable aposematically coloured food items, and suggests that pyrazine may also prolong memorability of this learned avoidance under certain circumstances. However, the conditioned and unconditioned stimuli need to be of a low intensity in order for this to be observable, as was noted in Experiment 5 and is discussed by Rescorla and Wagner (1972) and Pearce (1997).

There has been much debate about the mechanism through which visual conspicuousness enhances learned avoidance. Gittleman and Harvey (1980) argued that conspicuous prey are more easily detectable and therefore endure a higher initial rate of attack by naïve predators, which may account for the increased rate of learned avoidance observed with conspicuous prey. In our experiments, the relatively more conspicuous, aposematically coloured prey that smelt of pyrazine were attacked less frequently than the odourless prey, so any difference in the rate of learning was due to the characteristics of the warning display and not the encounter rate.

There was an observable difference in the rate of learning between chicks in the odourless and pyrazine treatments in Experiment 6, while no such difference was evident in Experiments 5 or 7. However, the total number of aposematically coloured crumbs attacked was lower in the presence of pyrazine than in its absence in all three experiments reported in this chapter, which adds weight to the argument that pyrazine enhances learned avoidance of both yellow and red prey.

Chicks in Experiment 6 showed evidence of consolidating their learned avoidance during the 3 hour interval after the last learning trial; however, chicks in Experiment 7 showed no such behaviour. This lack of consolidation suggests that red is a more salient signal, causing the chicks in all treatments in Experiment 7 to learn to
avoid the crumbs by the last learning trial. Conversely, in Experiment 6 the pale yellow may have been a less salient signal, and therefore only chicks in the pyrazine treatment learned to avoid it fully by the last learning trial, while chicks in the odourless treatment needed some extra time for the learned avoidance to consolidate. It may also be that these differences were caused by differences in the contrast with the background of the particular colours used in these experiments; this will be discussed in more detail in Section 8.2.3.

Pyrazine odour prolonged the memorability of the learned avoidance to beyond 96 hours, as the chicks in Experiment 6 showed signs of forgetting their learned avoidance of the odourless pale yellow crumbs, but the presence of pyrazine prolonged their memory. The chicks in Experiment 5 and 7 showed no signs of forgetting their learned avoidance of the odourless aposematically coloured crumbs. Therefore, the effect of pyrazine on memory could not be determined in these instances. These results suggest that both bright yellow and red may be more memorable signals than pale yellow. The pyrazine odour may have been observed to have an effect on memorability of bright yellow and red crumbs if they had been tested over a longer retention interval.

Speed (2000) pointed out that to date no studies have examined how two signals of a multimodal display interact with respect to memorability. The design of Experiments 6 and 7 sought to address this issue by allowing the chicks to learn to avoid the aposematically coloured crumbs that smelt of pyrazine and then comparing their memory of this learned avoidance in the presence of only the colour cue, or both the colour and odour cue used in the multimodal signal.

The results showed that both components of the multimodal display needed to be present in order for the avoidance to be fully remembered after the 96-hour retention interval (Experiments 6 and 7). This suggests that the pyrazine odour did not merely
serve to potentiate learning about the unpalatable yellow prey, but also served as an alerting signal as suggested by Rothschild and Moore (1987) or an *aide memoire* (Rothschild 1984).

In Experiment 6 when the number of yellow crumbs attacked by chicks in the four treatments during the 96-hour extinction trial was examined, a non-significant trend was observed. It suggested that learning to avoid a conspicuous visual signal in the presence of an odour cue may prolong memorability, even if the odour component of the signal is no longer present at the time of memory recollection. Similarly, when Roper and Redston (1987) offered their chicks red odourous prey in the learning trials and then odourless red prey in the extinction trials, the chicks showed gradual forgetting over time, but still appeared to recall some level of avoidance up to 72 hours after learning. Further work is needed in order to determine whether additional protection is to be gained by signalling using both components of a multimodal display and how this benefit changes as forgetting progresses.

The chicks appeared to generalise their learned avoidance of the yellow crumbs to the green crumbs in Experiment 6; however, the presence of the pyrazine odour appeared to enhance the chicks’ ability to differentiate between the two colours. The chicks also generalised between the bright yellow and bright green crumbs in Experiment 5, but the pyrazine odour did not alter this generalisation after the first learning trial. This may have been because the chicks were wary of the bright green as well as the bright yellow (see Section 5.2.4). No evidence of generalisation between the red and green crumbs was observed in Experiment 7. These results suggest that chicks are better able to differentiate between red and green crumbs than between yellow and green crumbs, and that pyrazine odour can reduce generalisation in the instances in which it occurs.

Fink and Patton (1952) and Heineman and Chase (1970) both noted that when
two signals differ through more than one sensory modality the chances of generalisation are reduced; therefore, animals may respond to colours in a different manner when an accessory signal such as an odour is presented. London (1954) suggests that olfactory or auditory stimuli may affect generalisation between colours in humans. The generalisation result from Experiment 6 suggests that palatable prey which are visibly different from the defended prey may not gain protection from close proximity to multimodal aposematic prey if the defended prey utilise pyrazine odour as part of their warning signal, since under these conditions visual discrimination is enhanced.

The experiments from this chapter provide evidence that pyrazine odour enhances avoidance learning of both red and yellow food. In the instances where forgetting occurred, pyrazine prolonged memorability, and it appears that in order for a multimodal signal to be remembered fully both component cues of the signal need to be present. Finally, in the instances where the learned avoidance was generalised to the palatable crumbs, the presence of pyrazine odour appeared to reduce this generalisation.
The effect of pyrazine odour on learned avoidance and memory in robins

Acknowledgement and authorship

The experiment reported in this chapter was designed, executed and analysed by E. Siddall. Dr. Marples, Dr. Speed and Dr. Rowland contributed to the design of the experiment. This experiment has been prepared as the manuscript “The effect of pyrazine odour on avoidance learning and memory in wild robins.” for submission to The Biological Journal of the Linnean Society.
EXPERIMENT 8 – The effect of pyrazine odour on learning and memory in wild birds

6.1 – INTRODUCTION

Chicks have been used as model avian predators by many researchers (Marples and Roper 1996; Rowe and Guilford 1999a; Marples and Kelly 1999; Skelhorn and Rowe 2005). Kelly and Marples (2004) noted that chicks’ responses to defended prey are often analogous to that of other bird species, but care needs to be taken when generalising behaviours observed with chicks in the laboratory to the responses of wild birds, as discussed in Section 1.6.1. As Experiment 6 was the first to show that pyrazine could enhance the rate of learned avoidance and memory of unpalatable yellow prey, it was decided that this should be tested using wild European robins (Erithacus rubecula). The experiment presented in this chapter sought to investigate whether wild robins exhibited similar behavioural responses to those shown by domestic chicks in the laboratory.

6.2 – METHODS

6.2.1 - Test subjects

The experiment was carried out using 16 wild European robins in Archbishop Ryan Park, Dublin 2, Ireland between October 2006 and January 2007. Robins were chosen as model predators as there was a large population in Archbishop Ryan Park. They were easily trainable, could be ringed using individually identifiable colour rings, and held individual territories, thus ensuring that individual robins could be tested in
isolation. Work of this manner had previously been conducted using robins (Marple et al. 1998; Thomas et al. 2004). The Archbishop Ryan Park is 4.75 hectares of managed public parkland; therefore, birds in the area were used to close proximity to humans, which facilitated direct observation of foraging decisions.

Robins have a broad insectivorous diet and may therefore be good representatives of how birds make foraging decisions about insect prey (Cramp 1988, Thomas et al. 2003). Prior to testing, the robins were caught using mist nets. The trapping was carried out under Dr. Marple’s license (BTO license, number F/CF/4601 and Irish NPWS license, R (B) 17/2006) and each bird was given an individually recognisable combination of colour rings on its left leg. During this time the winter territories of each individual were also mapped, to ensure that the experimental arena was placed in an area where only one robin would come to the tray. As they were winter territories, only one individual was present at each site, rather than a pair, as occurs in the summer territories (Cramp 1988).

6.2.2 - Artificial prey

Uncooked pastry baits were used as artificial prey. The pastry was made using 70 g flour: 30 g lard: 10 ml distilled water and dye solution (Marple et al. 1998; Thomas et al. 2004). This was rolled out to a thickness of 1.5 mm and then cut into small rectangles, to make baits which were 5 mm x 2.5 mm x 1.5 mm in size.

a) Colour cues

Commercial food dyes were used to colour the pastry, 1 ml of Sugarflair Colour Ltd.™ Egg Yellow or Spruce Green dye was diluted to make 90 ml of solution using distilled water, and then 10 ml of this solution was added to the flour and lard mixture
to make the pastry. The yellow baits were made unpalatable by dipping them in a solution of one part 2.5% W/V Denatonium benzoate (Macfarlan Smith Ltd.), commercially available as “bitrex”, to eight parts distilled water, and allowing them to dry overnight prior to testing.

b) Odour cues

The concentration of pyrazine solution was the same as that used in the laboratory experiments (Section 2.2.3), 100 µl of 2-isobutyl-3-methoxypyrazine diluted to 1000 ml using distilled water. In the odour treatment the pyrazine solution was placed on filter paper beneath the yellow baits, and distilled water was placed beneath the green baits. In the odourless treatment distilled water was placed beneath all baits.

6.2.3 - Experimental arena

The experimental arena was a black plastic tray measuring 35 cm long x 21 cm wide x 5.5 cm deep (Fig. 6.1) with white paper on the floor, so that both bait colours appeared equally conspicuous against the background (Thomas et al. 2004). One standard size (90 mm diameter) plastic Petri dish was placed in its lid in each corner of the tray. Each Petri dish was perforated with a radial pattern of holes in its base. The lid of the dish on which the Petri dish sat contained a piece of filter paper soaked in either the pyrazine odour solution or distilled water. The Petri dish and the base were separated using spacer pads. This prevented the transfer of the odour or water onto the baits, which might otherwise have affected the palatability of the baits. The holes in the base of the Petri dish allowed the odour to permeate up from the filter paper and be smelt by the bird as it ate the pastry baits. The Petri dish was sanded on the underside and painted white, so that the tray blended in with the base of the experimental arena and provided a contrasting background against which the baits were presented.
During pre-training, mealworms (*Tenebrio molitor*) were put into each of these four Petri dishes. The pre-training method is discussed further in Section 6.2.4. During the learning and memory trials the robins were given a choice test between palatable green and unpalatable yellow baits. Four baits of the same colour were placed in a cruciform pattern in each Petri dish, and Petri dishes containing the same colour of baits were placed in opposite corners of the feeding tray (Fig. 6.1). The robins were therefore offered a total of eight green and eight yellow baits during each trial. A small Petri dish (35 mm diameter) with one mealworm was placed in the centre of the experimental arena, to ensure that each robin came into the arena at the start of the learning trial. See below for details of the experimental arena.

6.2.4 - Pre-training

Each bird was pre-trained using mealworms to come to a fixed feeding site within its own territory. The feeding tray was placed at the feeding site with one mealworm in each of the four Petri dishes. Once the bird came down to feed, a signature whistle was given and repeated while the bird remained at the tray. This pre-training process was continued over a six week period until the robins readily came to the feeding tray upon hearing the signature whistle and were tame enough to continue to forage while allowing close observation of their food choice.
Figure 6.1: Bait arrangement and Petri dish position in the feeding tray for the wild bird experiment.
6.2.5 - Learning trials

Once the robins had completed their pre-training they were split into two treatment groups (Table 6.1), one in which all the baits were odourless and another where the yellow baits smelt of pyrazine. There were nine replicate individuals in the odourless treatment and ten in the pyrazine treatment. More robins were pre-trained than took part in the experiment, as several individuals disappeared during the course of the experiment. The Archbishop Ryan Park was divided into quarters with robins from diagonally opposite quarters of the park sharing the same treatments, so as to minimize any effects of territory location on the results obtained.

Table 6.1 Treatments received during the learning and extinction trials, indicated as bait colour and odour. Yellow baits were unpalatable during the learning trials.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>learning trials</th>
<th>extinction trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green; yellow</td>
<td>Green; yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green; yellow with pyrazine</td>
<td>Green; yellow with pyrazine</td>
</tr>
</tbody>
</table>

The robins were offered a choice test between eight palatable green baits and eight unpalatable yellow baits. The experimental arena was left in place for ten minutes, or until all the baits of one colour had been eaten. The number of yellow and green baits attacked during each trial was noted. The robins were given seven learning trials over the course of a week, with one trial performed in each territory between 8am and 11am each morning. The position of the Petri dishes was rotated one place anticlockwise each day, so that the robins could not learn to associate the position of the baits on the tray with their palatability, which may have affected their food choice. This also removed any effects that a favoured approach direction may have had on food choice.

A trial was considered to be a learning trial if the robin came to the experimental arena and ate the mealworm. Only robins that attacked at least one
yellow bait during the first learning trial were included as experimental subjects. This excluded two individuals from the odourless treatment and one individual from the pyrazine treatment, resulting in seven replicates in the odourless treatment and nine in the pyrazine treatment.

The learning trials were not conducted in wet or windy weather, as rain may have washed the bitrex off the baits and therefore affected palatability or diluted the pyrazine odour. The wind may also have blown the pyrazine odour around the experimental arena, thus reducing the odour gradient between the odour and non-odour dishes. Finally, wind tended to cause the feeding trays to overturn, making it difficult to conduct the experiment during adverse weather conditions.

6.2.6 - Memory trials

Once the seven learning trials were complete, the memory trials were conducted. The first was conducted after 96 hours, the next one week later, and the last one month after that. During the memory trials the green baits were palatable, but the yellow baits were unpalatable. These memory trials were therefore by definition not extinction trials, as an extinction trial is one that tests for the presence of the conditioned response (in this case avoidance of the yellow bait) in the absence of the conditioned stimulus (in this case the bitrex) (Pearce 1997).

This type of memory test was done because no studies have yet shown how long wild birds remember learned avoidance and so it was necessary to be able to test the same individual multiple times after different retention intervals. If a traditional extinction trial had been conducted the birds may have formed an association between the yellow baits and palatability, which would have eroded their learned avoidance, and affected subsequent memory tests.
6.2.7 – Data analysis

Most of the data were non-normal and not transformable by any standard method, therefore non-parametric statistics were used for the analysis (Zar 2005). A Kruskal-Wallis test was conducted to determine whether the number of yellow baits attacked differed across the seven learning trials and therefore whether avoidance learning occurred. A Mann-Whitney U test compared the number of baits attacked in the first and last learning trials. In addition the mean number of yellow baits attacked by robins in each treatment group was calculated, these data were normal for the number of yellow baits attacked, therefore a t-test was used to analyse the difference between the odour and non-odour treatments. However, the response of the robins towards the green baits were non-normal and non-transformable therefore a Mann-Whitney U test was used for the analysis. The total number of yellow baits attacked by each chick in the two treatments was also compared using a Mann-Whitney U test, to determine whether yellow baits that smelt of pyrazine were better protected than the odourless yellow baits, in a type of analysis similar to that conducted by Skelhorn and Rowe (2006). The number of yellow baits attacked during the first and last learning trials, and the three memory trials were compared using a Kruskal-Wallis test, to determine whether the pyrazine odour had any affect on memorability of the learned avoidance.
In contrast with the clear learned avoidance of yellow crumbs that smelt of pyrazine shown by domestic chicks in the laboratory (Experiment 6), there were no significant differences between the number of yellow baits attacked across the seven learning trials in either the odourless (Kruskal-Wallis test, $\chi^2 = 3.768, d.f. = 6$, NS; Fig. 6.2) or pyrazine treatments (Kruskal-Wallis test, $\chi^2 = 4.521, d.f. = 6$, NS; Fig. 6.2).

There were also no significant differences between the number of yellow baits attacked during the first and last learning trial by robins in either the odourless (Mann-Whitney U test, $U = 12.50, n = 7, 7$, NS) or pyrazine (Mann-Whitney U test, $U = 24.00, n = 9, 9$, NS) treatments, which implies that neither group learned to avoid the unpalatable yellow baits by the seventh learning trial.

However, when the mean number of yellow baits attacked in each trial was compared between treatments (Fig. 6.2), it was noted that in all learning and memory trials robins in the pyrazine treatment attacked significantly fewer yellow baits than robins in the odourless treatment ($t$ test, $t (18) = 4.29, p < 0.001$; Fig. 6.2), suggesting that the pyrazine odour increased the robins' avoidance of the yellow baits.
There were no significant differences in the number of green baits attacked across the seven learning trials by robins in either the odourless (Kruskal-Wallis test, $\chi^2 = 8.605$, $d.f. = 6$, NS; Fig.6.3) or pyrazine treatments (Kruskal-Wallis test, $\chi^2 = 0.549$, $d.f. = 6$, NS; Fig. 6.2). And there was no significant difference between the number of green baits attacked during the first and last learning trial by birds in either the odourless (Mann-Whitney U test, $U = 16.50$, $n = 7,7$, NS) or pyrazine (Mann-Whitney U test, $U = 36.00$, $n = 9,9$, NS) treatments.

When the means of the number of green baits attacked in each learning trial were analysed it was noted that after trial two, robins in the pyrazine treatment attacked
significantly more green baits than robins in the odourless treatment (Mann-Whitney U test, $U = 4.50$, $n = 6, 6$, $p < 0.05$; Fig. 6.2). In the first trial there was no significant difference between the treatments in the robins' willingness to attack the green baits. This suggests that the robins' innate response towards green food was not affected by the presence or absence of the pyrazine odour associated with yellow baits. After the second learning trial the greater consumption of green baits in the pyrazine treatment suggests that the presence of the pyrazine odour associated with the yellow baits made the birds aware that the green baits were palatable.

There were no significant differences in the number of yellow and green baits attacked by robins in the odourless treatment until trial 4 at which point the birds attacked significantly more yellow than green baits (Mann-Whitney U test, $U = 3.50$, $n = 7, 7$, $p < 0.01$; Fig. 6.2), this difference was maintained for the rest of the study with the exception of learning trial 5, and the 96-hour memory test. This suggests that the birds in the odourless treatment were more willing to attack the yellow than the green baits even though the yellow baits were unpalatable.

Robins in the pyrazine treatment attacked the same number of green and yellow baits throughout the learning and memory trials (Fig. 6.2). This suggests that in the presence of the pyrazine odour the birds did not differentiate between the two colours despite the yellow being unpalatable and the green palatable.

When the total number of yellow baits attacked across all trials were examined, it was noted that the robins in the pyrazine treatment attacked fewer yellow baits than robins in the odourless treatment (Mann-Whitney U test, $U = 12.50$, $n = 7, 9$, $p < 0.05$; Fig. 6.3). These data reflect a similar trend to that observed with chicks in Experiment 6 (Fig. 5.3.2).
Figure 6.3: The mean of the total number (±s.e) of yellow crumbs attacked during all trials in which yellow prey were unpalatable by robins in the odourless and pyrazine treatments.
Figure 6.4: The mean number (±s.e) of yellow baits attacked in the first and last learning trials, and the 96-hour, 1 week and 1 month memory trials.

There were no significant differences between the number of yellow baits attacked during the first and last learning trial and the three memory trials by robins in either the odourless (Kruskal-Wallis test, $\chi^2 = 6.042$, d.f. = 4, NS; Fig. 6.4) or pyrazine (Kruskal-Wallis test, $\chi^2 = 3.140$, d.f. = 4, NS; Fig. 6.4) treatments, which suggests that pyrazine odour had no effect on memorability in this instance.
The pyrazine odour reduced the total number of yellow baits attacked by the robins during all the trials in which the yellow baits were unpalatable. This result is similar to the behaviour exhibited by chicks in the laboratory (Experiment 6). However, the robins in both the odourless and pyrazine treatments showed no significant reduction in the number of yellow baits they attacked across the seven learning trials. This result may have occurred as the robins were already avoiding the yellow baits in the first learning trial, and therefore a change in learning rate could not be recorded. It also may be that the robins did not acquire a learned avoidance across the seven learning trials. The pyrazine odour also made no difference to how the robins treated the unpalatable yellow baits during the memory trials. However, this result may be due to the fact that the robins did not learn to avoid the yellow baits rather than a lack of an effect of pyrazine on the memorability of a signal.

An additional analysis was conducted on the robin data; the mean number of yellow baits attacked by robins in the pyrazine treatment was compared to the mean number attacked by robins in the odourless treatment for each trial. It was noted that robins in the pyrazine treatment attacked significantly fewer yellow crumbs across all the learning and memory trials than robins in the odourless treatment. This further suggests that the pyrazine odour reduced the robins’ willingness to attack the unpalatable yellow baits in a similar manner to that observed with domestic chicks (Experiment 6). If birds’ responses to insects reflect those shown towards the pastry baits, then a toxic insect population that advertised its defended state using both a yellow visual display and pyrazine odour may be better protected than insects that used the yellow visual signal alone.
When the yellow baits were odourless the robins attacked more yellow than green baits despite the fact that the yellow baits were unpalatable. This result is somewhat surprising, and may be due to a greater level of contrast between the green baits and the white background of the feeding tray, than the yellow baits and the white background. When the pyrazine odour was associated with the yellow baits the robins reduces their number of attacks on the yellow baits suggesting that the pyrazine odour enhanced their awareness that the yellow baits were unpalatable. Robins in the pyrazine treatment also attacked more green baits than robins in the odourless treatment which suggests that the pyrazine odour may have alerted the robins to the palatable of the green baits. These data support Rothschild and Moore (1987) suggestion that pyrazine may act as an alerting stimulus, thus causing the predator to pay more attention to their predatory decisions.

The differences between these robin data and the chick data from Experiment 6 may be due to several factors. The aversive substance, bitrex, may have been too strong, and therefore the robins formed an avoidance of the baits in the first learning trial. This would explain why the robins did not appear to learn an avoidance during the subsequent learning trials, in a similar manner to that noted for Experiment 5, Section 5.2.4. It may also be that the bitrex was not strong enough, and therefore no learned avoidance was acquired which would explain why pyrazine did not appear to affect the memorability of the signal.

The chicks in the laboratory were food deprived for a set period of time which standardised their hunger levels; however, this was not possible with the wild birds, and differing levels of hunger may have introduced a greater level of variability into the data. Wild birds may be willing to eat more unpalatable prey than laboratory held birds because they are hungrier. In addition, the sample size of robins was necessarily far smaller than for chicks, and a greater sample size may have made the trends
observed from the robin data statistically significant. The domestic chicks used in Experiment 6 originated from the same batch of chicks and therefore may have had low genetic variability, which may have made their behaviour quite uniform. The wild population of robins used in this study may, on the other hand, have large genetic variability which could have contributed to the variability in the behaviour observed (Marples and Brakefield 1995). The wild birds may also have had more distractions than the chicks, such as predator avoidance and territory defence. This may have caused the robins to pay less attention to their foraging decisions than the chicks. Finally, the yellow visual cue and the pyrazine odour were novel to the newly hatched chicks; however, the wild birds may have encountered one or both of these cues before, which may have decreased their reaction to the multimodal signal.

Given the many reasons one might expect differences between a bird foraging in the wild and a chick foraging in the laboratory, the results from the wild birds reflect those of the laboratory chicks remarkably closely. The results demonstrate that pyrazine odour enhances avoidance of unpalatable yellow prey by wild robins. This is the first demonstration of such behaviour by wild birds, and adds weight to the argument that a toxic insect that advertises its defended state using both a yellow signal and pyrazine odour might well be better protected than an insect using a yellow visual signal alone.
CHAPTER 7

The effect of buzzing of *B. terrestris* on learned avoidance and memory in chicks

Acknowledgement and authorship

The experiment reported in this chapter was designed, executed and analysed by E. Siddall. Dr. Marples contributed to the design of the experiments. This experiment is part of the manuscript “Hear no evil: The effect of auditory warning signals on avian innate avoidance, learned avoidance and memory” which is in preparation for submission to *Behavioral Ecology.*
EXPERIMENT 9 – The effect of buzzing of *B. terrestris* on learned avoidance and memory of yellow crumbs.

7.1 – INTRODUCTION

The results from Chapter 5 suggested that pyrazine odour can enhance avoidance learning and prolong memory. Rothschild (1984) proposed that warning sounds may have a similar effect on predatory decisions. Rowe (2002) found that an artificial beeping noise reduced the number of trials needed for chicks to learn a discrimination between rewarded and unrewarded prey, but this study used colours and sounds not naturally found as warning signals, and therefore may not reflect how avian predators respond to defended insect prey. Hauglund *et al.* (2006) found no such effect of the flying buzzing sound of *D. media* on the mean avoidance across all learning trials of unpalatable yellow prey, but did see some evidence of increased learned avoidance of unpalatable striped green and black prey in the presence of buzzing.

Neither Rowe (2002) nor Hauglund *et al.* (2006) investigated whether the auditory signal increased encounter rate, or directly addressed whether sound affects the rate of avoidance learning. The learning experiment presented below was therefore designed to address both these questions. Hauglund *et al.* (2006) is thus far the only empirical investigation of how a warning sound affects memory in avian predators. They found no effect of buzzing on memorability of the learned avoidance, and noted that, if anything, buzzing appeared to speed up forgetting.

This current experiment directly assessed whether buzzing of *B. terrestris* affected the rate at which a learned avoidance was acquired. The effect buzzing had on memorability was also examined in more detail.
The experiment was conducted over one week with 25 chicks in each of the two treatments (Table 7.1). The pre-training was carried out on day one, as described in Section 2.4. On day two the chicks were offered a three-minute learning trial with a choice test between 12 unpalatable yellow and 12 palatable green crumbs in the presence or absence of the buzzing sound (Table 7.1). Both the crumb colours were novel to the chicks. The learning and extinction trials were conducted in the same manner as reported for Experiment 6 (Section 5.3.2). There were 13 chicks per treatment for the 3-hour extinction group and 12 chicks per treatment for the 96-hour extinction group.

Table 7.1: Treatments received during the learning and extinction trials, indicated as crumb colour and sound. Yellow crumbs were unpalatable in the learning trials. All crumbs were palatable in the extinction trials.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>learning trials</th>
<th>extinction trials (3 hours or 96 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Green; yellow</td>
<td>Green; yellow</td>
</tr>
<tr>
<td>2</td>
<td>Green; yellow with buzzing</td>
<td>Green; yellow with buzzing</td>
</tr>
</tbody>
</table>
Chicks in both treatments learned to avoid the unpalatable yellow crumbs by the seventh learning trial (Mann-Whitney U test, Treatment 1, trial 1 vs. 7; $U = 134.00$, $n = 24$, $p < 0.001$; Treatment 2, trial 1 vs. 7; $U = 147.50$, $n = 24$, $p < 0.01$; Fig. 7.1). There were no significant differences between the numbers of yellow and green crumbs attacked by the two treatment groups during any of the learning trials, as Kruskal-Wallis tests for each trial yielded no significant results (see Fig. 7.1).

![Graph showing mean number of yellow and green crumbs attacked across seven learning trials.](image)

**Figure 7.1:** The mean number of yellow (triangles) and green (squares) crumbs attacked across the seven learning trials by chicks in the soundless treatment (solid line) and chicks in the buzzing treatment (dashed line). Standard error bars have been omitted for clarity.

There were no significant differences between the treatment groups in the number of yellow crumbs attacked in any of the learning or extinction trials, which
suggests that the presence of buzzing had no effect on innate avoidance behaviour, the extent of learning, or memorability of the learned avoidance after 3 hours or 96 hours (see Fig. 7.1 and 7.2).

Chicks in both the buzzing and silent treatments attacked significantly different numbers of yellow crumbs during the first and last learning trials and the two extinction trials (Kruskal-Wallis test, Treatment 1, $\chi^2 = 21.11$, d.f. = 3, $p < 0.001$; Treatment 2, $\chi^2 = 14.12$, d.f. = 3, $p < 0.01$; Fig. 7.2), suggesting that chicks in both treatments acquired the learned avoidance of the unpalatable yellow crumbs.

Chicks in the silent treatment showed no evidence of further consolidating their learned avoidance during the 3-hour consolidation interval, as there was no significant difference between the number of yellow crumbs attacked during the last learning trial and the three-hour extinction trial (Dunn’s post-hoc test trial 7 vs. 8 NS; Fig. 7.2), although the graph does suggest some trend towards a consolidation effect. Chicks in the buzzing treatment appeared to forget their learned avoidance to an intermediate level, as the 3-hour retention interval trial was not significantly different either to the first learning trial (Dunn’s post-hoc test trial 1 vs. 8 NS; Fig. 7.2), or to the last learning trial (Dunn’s post-hoc test trial 7 vs. 8 NS; Fig. 7.2).

After the 96-hour retention interval the chicks in both treatments had forgotten their learned avoidance of the unpalatable yellow crumbs and reverted to the same level of attack as in the first learning trial (Dunn’s post-hoc test; Treatment 1 trial 1 vs. 9 NS; Treatment 2 trial 1 vs. NS, Fig. 7.2).
Figure 7.2: The mean number (±s.e) of yellow crumbs attacked by the soundless and buzzing treatments in the first and last learning trials, and the 3-hour and 96-hour extinction trials.

The total number of yellow crumbs attacked across all seven learning trials did not differ between the treatments (Mann-Whitney U test, $U = 282.50$, n = 24, 24, NS; Fig. 7.3), which again suggests that buzzing did not enhance avoidance of the yellow crumbs throughout the entire experiment.
7.4 – DISCUSSION

The presence of agitated buzzing had no effect on learned avoidance of unpalatable prey. As the learning trials progressed, chicks in the two treatments learned to avoid the yellow crumbs at the same rate, which suggests that the agitated buzzing of *B. terrestris* had no effect on avoidance learning in these chicks. Hauglund *et al.* (2006) found that the buzzing of a flying wasp had no effect on mean avoidance learning of yellow prey; however, they did report some effect with striped green and black prey. Rowe (2002) found that an artificial beeping noise decreased the number of trials needed for chicks to learn to discriminate between rewarded and unrewarded artificial baits; however, this experiment used colours and sounds not classically considered to be warning cues, and may therefore not reflect how birds would respond to natural aposematic insect prey. These differences may also have been due to
differences in the protocol between this and the Rowe (2002) experiment. Rowe (2002) had 20 to 21 birds in each treatment, while this experiment had 12 to 13. Their results suggest that the behavioural responses of chicks can be altered by an auditory signal, but it remains to be seen whether similar results can be obtained using a naturally occurring sound.

Van Kampen and Bolhuis (1991) noted a greater effect of natural versus artificial auditory signals on imprinting, which suggests that if certain insect sounds operate as warning signals they may very well improve avoidance learning in a similar manner and possibly to a greater degree than that observed by Rowe (2002) with the artificial beep.

Hauglund et al. (2006) noted that the presence of buzzing appeared to speed up forgetting of the learned avoidance. Our results suggest a similar effect, as chicks in the buzzing treatments showed some signs of forgetting their learned avoidance after the 3-hour retention interval while chicks in the silent treatment showed no such effect. There was no significant evidence of consolidation after the 3-hour retention interval, contrary to previous observations (Experiment 6). This suggests that consolidation may be quite a variable process. It may be that the chicks in Experiment 9 learned faster than the chicks in Experiment 6, and therefore consolidation was not observable after the 3-hour retention interval.

Buzzing did not prolong memorability over the more extended 96-hour retention interval, as the chicks in the silent and buzzing treatments forgot their learned avoidance to the same extent. Hauglund et al. (2006) suggested that buzzing may facilitate eating; however, the current results show no such effect, as there were no differences between the total number of yellow crumbs attacked by the two treatments during the learning trials. There were also no differences in the number of yellow crumbs attacked during the extinction trials.
If these results are a proxy for how wild birds respond to insect prey, then they suggest that insects such as bees and wasps would gain no extra protection against bird predation by producing a buzzing sound. These results are somewhat surprising given reports that hoverflies mimic not only the visual component but also the auditory component of their hymenopteran model’s display (Gaul 1952; Brower and Brower 1961), which would imply that some additional benefit is to be gained from producing a buzzing sound. The present results suggest that this benefit may not be related to reducing predation by avian predators; however, previous workers on the area of auditory signals do not clarify whether the buzzing produced by hoverflies mimics the flying buzzing or agitated buzzing of their hymenopteran mimics (Carpenter and Ford 1933; Myers 1935; Gaul 1952; Brower and Brower 1961). Golding et al. (2001) noted that the frequency of the buzzing produced during flight by a range of hoverfly species overlaps with that of bumblebees and honeybees (*Apis mellifera*).

There is evidence of buzzing acting as a warning signal to non-avian predators. Elephants may actively avoid the buzzing sound of African honeybees (*Apis mellifera scutellata*) (King et al. 2007). Carpenter and Ford (1933) reported that after a brief handling, a monkey dropped a dronefly as if it had been stung. They suggest that this response may have been caused by the buzzing sound. Spiders appeared to draw back from hoverflies upon hearing their buzzing sound (Myers 1935). Brower and Brower (1961) observed that toads avoided hoverflies with greater frequency when they had their wings and could buzz than when their wings were absent, and suggested that auditory as well as visual mimicry may be important for protection of the hoverfly.

It was surprising that the chicks did not respond to the buzzing sound, as Kirchner and Roschard (1999) recorded the buzzing of *B. terrestris* to be 45 – 55 dB between 1 kHz and 6 kHz, and the buzzing used in this experiment had a maximum volume of between 87 dB to 95 dB between frequencies of 1 kHz and 5 kHz, which is
well within the hearing range of birds (Dooling 2004). Despite being able to hear the auditory cue and appearing to respond when the buzzing was played, the predatory decisions of the chicks were not altered by buzzing. It may be that for birds, buzzing does not act as a warning signal. Clark et al. (1993) proposed that just because a receiver can detect an odour does not mean that signal will have an effect on its behaviour, and our results suggest a similar phenomenon with auditory cues.

There are many examples of bird behaviour being altered by a multimodal signal comprised of both a visual and an auditory signal. Visual imprinting in chicks may be improved when an auditory cue is presented simultaneously with the visual stimulus (Van Kampen and Bolhuis 1993). There is ample evidence that birds are capable of using multimodal signals with auditory and visual component cues for learning (Hultsch et al. 1999; Kilner et al. 1999). Insects produce sounds within the hearing range of birds, and there is evidence that birds are responsive to auditory cues; therefore, it seems likely that birds may attend to acoustic warning displays.

There is a plethora of insect sounds associated with warning displays that may operate as warning sounds, such as stridulation in bees and beetles that has been noted to reduce predation by wolf spiders and mice (Masters 1979), and the high pitched clicking of certain moth species that has been noted to deter predation by bats (Dunning 1967). This experiment showed that, rather surprisingly, chicks do not use a biologically relevant sound cue to help them learn to avoid distasteful prey. Although chicks could certainly hear the stimulus, it was not considered salient in their decision-making process. Further work into how these other potential warning sounds affect avian predator behaviour will provide an interesting avenue for further research.
CHAPTER 8 – GENERAL DISCUSSION
GENERAL DISCUSSION

Avian avoidance behaviour of insect warning signals is enhanced by the presence of pyrazine odour (Experiments 1, 2, 4, 5, 6, 7 and 8); however, an agitated buzzing sound had no such effects (Experiments 3, 4 and 9). If these results reflect the responses of wild birds to natural prey types, then this may suggest that there are benefits to aposematic insects advertising their defended state multimodally, but that the effects of the multimodal signals are dependent on the component cues. In the case of pyrazine odour there appears to be no trade-off between signals that enhance innate avoidance behaviour and those that speed up learning and retard forgetting as suggested by Braveman and Jarvis (1978) and Miller and Holzman (1981).

8.1 – THE EFFECT OF MULTIMODAL SIGNALS ON AVOIDANCE BEHAVIOUR

8.1.1 - Innate avoidance responses to multimodal signals

Pyrazine odour, as part of a multimodal signal, was observed to increase both neophobia and dietary conservatism towards novel yellow (Experiments 1 and 4) and novel red crumbs (Experiment 2). Buzzing of *B. terrestris* did not alter innate avoidance behaviour (Experiments 3 and 4). Familiarity with either the odour or colour component of the multimodal display had different effects on innate avoidance behaviour of the chicks. This suggests that contextual isolation (see Section 1.3.4), may be dependent on the component cue in question. When chicks were familiar with the yellow signal they showed a reduction in both neophobia and dietary conservatism towards the multimodal (yellow and pyrazine) signal. However, Rowe and Guilford
(1999a) observed that pyrazine could elicit avoidance of yellow as long as the chicks were not overly familiar with the visual cue. This suggests that contextual isolation of the visual signal only occurs when the colour cue is relatively unfamiliar.

On the other hand, the predator’s familiarity with pyrazine odour did not affect the odour’s ability to prolong both neophobia and dietary conservatism of novel yellow crumbs, suggesting that contextual isolation occurs with the odour cue. It may be that chicks forget their experience with the pyrazine odour during the interval between the familiarisation and test trials (see Section 3.1.4) thus treating familiar and novel pyrazine in the same manner. The ability of pyrazine to prolong the memory of a learned avoidance, as observed in Experiment 6, argues against this interpretation of the data. However, the pyrazine odour in Experiment 6 was associated with unpalatable crumbs and a warning colour. It is possible that when the odour was presented with palatable, familiar crumbs (as in the pre-training trials in Experiment 1) experiences with the odour were more quickly forgotten. It may also be that because vision is a more dominant sense for birds than olfaction (see Section 1.3), a novel colour cue can elicit a response in the presence of a familiar odour but not the other way around.

8.1.2- Learned avoidance and memory of multimodal signals

Pyrazine also enhanced learned avoidance of unpalatable red prey by chicks (Experiment 7) and unpalatable yellow prey by chicks (Experiments 5 and 6), and robins (Experiment 8). This is the first demonstration of pyrazine enhancing avoidance learning of solid prey. It is also the first piece of evidence to suggest that pyrazine odour can enhance learned avoidance behaviour in wild birds (Experiment 8) as well as domestic chicks. The agreement of the results between Experiments 6 and 8 suggests
that despite the differences between laboratory tested neonatal chicks and adult wild robins, chicks are a good initial model with which to study the effect of insect warning displays on avian foraging behaviour.

Gittleman and Harvey (1980) suggested that conspicuous prey may be detected and attacked more frequently than cryptic prey, and this may account for the faster learning rates observed with conspicuous prey. However, during the learning experiments the chicks attacked fewer multimodal than monomodal crumbs; therefore, the enhancement of avoidance learning must be due to some property of the warning signal that facilitates learning, rather than an increase in encounter rate.

In the experiments where forgetting occurred, the presence of the pyrazine odour prolonged the memory of the learned avoidance (Experiment 6). The results from Experiments 6 and 7 suggest that both component cues of a multimodal display composed of a colour and an odour need to be present in order for the learned avoidance to be remembered. This is the first demonstration of the interaction of component signals on memory, and suggests that pyrazine odour operates to enhance memory as well as learning.

Agitated buzzing did not have an effect on learned avoidance behaviour (Experiment 9). It is possible that buzzing may be a multimodal signal itself, which signals to the receiver in both auditory and tactile modalities. This may explain the lack of effect observed in the experiments detailed here, as only the auditory signal was presented. Also, buzzing may interact with other components of the hymenopteran warning display not tested in this thesis, such as the colour pattern, black colour, shape, taste, and movement. The buzzing sound was played through speakers at the side of the arena rather than from beneath the aposeatically coloured crumbs. If it could have been played from below, this would have reflected the presentation of insect warning signals more accurately, and may have caused the buzzing to alter behaviour.
Given the constraints of the experimental arena this was impossible to do, but it certainly warrants consideration in the design of future experiments.

If the results from these experiments reflect how wild birds respond to live insect prey, then several conclusions may be drawn. Insects would gain greater protection through both avian innate and learned avoidance behaviour if they possessed both a warning colour such as yellow or red, and a warning odour, such as pyrazine. The addition of an agitated buzzing sound did not enhance any form of avoidance behaviour measured; however, other warning sounds may produce observable effects. This suggests that there may be something special about pyrazine as a signal or odours as opposed to sounds, and that this effect would not be observed with just any additional cue. Creating general rules about avoidance behaviour is problematic, as the method of presentation (Speed 2000), the intensity of the signal, the use of live insects and wild avian predators may well change the behaviours observed. Nevertheless, the use of a model system such as that described here offers a good starting point for understanding how component signals of multimodal displays interact with one another.
8.2.1 – Are the component cues of warning signals unique?

The results discussed in Section 8.1 raise the question of whether the behavioural responses observed occur only in response to specific warning signals or whether the presence of any additional cue could elicit similar effects.

Pyrazine odour prolongs neophobia and dietary conservatism (Experiments 1, 2 and 4), and Marples and Roper (1996) argued that specific odours such as pyrazine may be unique in their ability to elicit innate wariness. They noted that innate avoidance behaviour towards novel coloured crumbs was enhanced by odours typically associated with warning displays (pyrazine and almond), but not by non-warning odours (vanilla and thiazole). This suggests that there may be something special about odours involved in insect warning displays, and not all novel odours would have the same effect. However, Jetz et al. (2001) tested the effect of several novel odours on innate avoidance behaviour and found that both warning (pyrazine) and non-warning odours (methyl salicylate and ethyl acetate) enhances innate bias against yellow to the same extent. They concluded that any odour may enhance innate biases as long as it is novel. In this experiment the odour was placed under every crumb (Jetz et al. 2001); as noted in Section 8.2.2, this may have caused the odours to act as alerting signals. Therefore any novel odour may be able to operate as an alerting signal, but only specific odours such as pyrazine may work as warning signals.

Pyrazine odour prolongs innate avoidance behaviour even when familiar (Experiment 1), which suggests that the birds are responding to more than just the novelty of the pyrazine. In order to test this further, additional experiments similar to Experiment 1 could be conducted to examine whether other odours must be novel in
order to elicit avoidance behaviour. If so, this suggests that there is something unique about pyrazine as an olfactory warning signal.

Pyrazine odour improves avoidance learning and retards forgetting (Experiment 6). Odours such as almond and vanilla have also been observed to enhance avoidance learning. Additionally, almond prolongs memory of this avoidance whilst vanilla does not (Roper and Marples 1997). Thus far there has been no empirical examination as to whether certain odours are better than others at facilitating learned avoidance behaviour. This could be tested using an experiment similar to Experiment 6 in which the effect on learning and memory of pyrazine and other odours, both warning and non-warning, could be examined.

If pyrazines are unique in their effects on avian avoidance behaviour, then this might suggest that defended insects evolved pyrazine as a warning signal, and subsequently avian predators evolved a response to this signal. Jetz et al. (2001) argues the contrary, as their results showed that any novel odour has the same effect as pyrazine, and suggest that avian predators first had a propensity to respond to odours, and subsequently defended insects evolved pyrazine in order to exploit this. However, warning signal evolution may pre-date vertebrate predators, and may have evolved initially in response to invertebrate predators (Rothschild and Moore 1987). Pyrazines represent an example of convergent evolution in warning displays, as they are found across so many animal and plant taxa (Rothschild and Moore 1987; Moore et al. 1990). They are also highly volatile, which gives them a low detection threshold (Guilford et al. 1987). Pyrazine odour may be present in forest fires; therefore, the coupling of this odour with two common warning colours from the flames, red and yellow, may explain why animals have propensity to attend to these specific cues so readily (Rothschild and Moore 1987).
Buzzing does not enhance either innate or learned avoidance behaviour (Experiments 3, 4 and 9), although, as already suggested, other insect warning sounds may have an effect. A preliminary experiment suggests that buzzing of a mosquito may enhance innate avoidance behaviour to the same level as observed with pyrazine (P. McAteer, Pers. Comm.). If other insect sounds have an effect, but buzzing does not this suggests that it is only specific sounds that enhance the avoidance behaviour of avian predators. This supports the idea that there are unique warning signals such as specific colours and odours, and maybe even specific sounds.

If specific warning signals exist, then it is important to use these signals when examining how avian predators respond to warning signals, as by using non-warning signals the true response may not be observed. Experiment 4 of this thesis examined the effect of a trimodal signal on innate avoidance behaviour; however, it used a combination of component cues not typically found in nature, which may explain why no effect was observed. Marples et al. (1994), on the other hand, examined the trimodal warning display used by the seven-spotted ladybird and observed interaction between the component cues.

8.2.2 - Are additional cues warning or alerting signals?

The enhancing effect of additional signals in a multimodal display observed in this thesis may be attributed to one of two effects (Marples and Roper 2004). The cue may act as a warning signal in its own right, or it may act as an alerting signal to the visual component of the multimodal display as suggested for odours (Rothschild 1984) and sounds (Claridge 1974). If all the component cues are warning signals, then each should elicit an innate avoidance response, and act as a learning cue independently of other components of the warning display (Marples and Roper 2004). If certain cues operate as alerting signals, then they should merely serve to alert the predator to the
warning signal (i.e. the colour cue) and enhance innate and learned avoidance towards this signal (Rothschild and Moore 1987). This is a psychological question about how the component signals operate to affect the receiver's psychology. Whether the signals operate as alerting or warning signals in their own right does not affect the ecological implications of how the predator responds to the multimodal warning display. Consideration of the detail of other researchers' experimental designs provides some insight into whether component cues operate as warning or alerting signals; however, additional work is needed in order to distinguish between these two effects conclusively.

The observation by Guilford et al. (1987) that pyrazine odour can act as a discriminatory learning cue in the absence of visual stimuli supports the warning signal hypothesis. However, in order to test this conclusively an additional experiment needs to be conducted. Chicks should be trained to avoid unpalatable yellow crumbs that smell of pyrazine, in a manner similar to Experiment 6. Once the learning trials have been completed the chicks could then be offered either the multimodal signal (yellow and pyrazine), the yellow signal alone, or the pyrazine odour alone in an extinction trial. This would allow comparison of the conditioned response towards the multimodal display and to each of the component signals. If avoidance is detected in response to the pyrazine odour alone this would suggest that the odour operates as a warning signal in its own right. If there is no avoidance of the pyrazine odour it may be that the odour acts as an alerting signal and potentiates the learned avoidance of the yellow crumbs, as Rothschild and Moore (1987) suggested. Such a result may also occur if the yellow signal overshadowed an association of the pyrazine odour with the unpalatable crumbs; however, this is unlikely to be the case as avoidance of the yellow pyrazine crumbs was greater than the yellow crumbs alone in Experiment 6.
There is a methodological problem in conducting the experiment suggested above. When using solid food, it is impossible to offer the pyrazine odour without a visual signal. Guilford *et al.* (1987) overcame this problem by testing whether chicks could learn to discriminate unpalatable water that smelt of pyrazine odour from palatable odourless water. A similar method could be used in conducting an experiment such as the one described above. However, as Roper and Marples (1997b) noted the response to liquid and solid prey often differs, therefore it is necessary that the liquid prey in this experiment be offered to the birds of the floor of the experimental arena rather than in a drinker.

The question of whether pyrazine operates as an alerting or a warning signal also needs to be investigated with regards to innate avoidance behaviour. Marples and Roper (1996) examined the effect of colour and odour on innate avoidance behaviour. They offered chicks novel coloured crumbs, novel coloured crumbs that smelt of novel pyrazine, and familiar coloured crumbs that smelt of novel pyrazine. They observed that the novel colour cue elicited innate avoidance behaviour and while the pyrazine odour enhanced the avoidance of the novel coloured crumbs, the pyrazine odour itself did not elicit any innate avoidance behaviour. From these results it could be concluded that pyrazine odour operates as an alerting and not a warning signal. However, the effect of odour was tested by measuring how much avoidance the odour elicited when paired with a familiar colour. As already discussed in Section 8.1.1, the familiarity of the colour cue may therefore have affected the response to the odour signal. An additional experiment along the lines of the methodology used by Guilford *et al.* (1987) could be used to examine this question in more detail, by comparing innate avoidance towards a novel yellow signal, a novel pyrazine signal, and a novel yellow, pyrazine signal. However, as previously discussed, caution must be taken in the manner with which the liquid prey is presented to the birds.
Rowe and Guilford (1999a, b) offered additional cues (pyrazine or buzzing) to their chicks in association with both the green and yellow crumbs. Their results suggest that, in the context of their experiments, the additional cues acted as an alerting rather than a warning signal. In the pyrazine experiment (Rowe and Guilford 1999a) with the odour present beneath both colours of crumb, it was not available as a second cue to discriminate palatable (green) and unpalatable (yellow) food. An effect of pyrazine on innate avoidance was still observed; therefore, the odour must have been alerting the chicks to the presence of the aposematically coloured yellow crumbs, towards which they showed an innate avoidance response.

The same holds true in the buzzing experiment (Rowe and Guilford 1999b). As buzzing was played for the entire time the chicks were in the experimental arena and it elicited an innate bias against the yellow crumbs; therefore, it must have functioned to alert the chicks to the presence of the yellow crumbs. In Experiment 3 the buzzing sounds was only played at brief intervals when the chicks were engaged with an aposematically coloured crumb. And although it did not have any effect on neophobia or dietary conservatism, it did enhance discrimination between the red and green crumbs. These results suggest that buzzing may have some alerting effect that enhances discrimination, but not innate or learned avoidance behaviour. Therefore, buzzing may function as an alerting signal to some extent, whereas pyrazine may act as a warning signal, but as already stated further investigation is needed in order to differentiate between the two hypotheses.

8.2.3 – The differences in visual signals

The chicks treated red as a more salient colour cue than yellow throughout the experiments. Familiarity with yellow crumbs deactivated dietary conservatism and affected how the odour and colour components of the multimodal signal interacted with
one another (Experiment 1). On the other hand, chicks appeared to be more resistant to familiarisation with red than yellow as familiarity with red did not deactivate dietary conservatism (Experiment 2). Resistance to familiarity may mean that red insects are better protected than yellow insects through innate avoidance, even by experienced predators.

The results from the learning experiments also show differences in the chicks’ responses to yellow and red crumbs. The chicks showed evidence of consolidation of the learned avoidance of yellow crumbs after the 3-hour retention interval (Experiment 6), but there was no such effect with red crumbs (Experiment 7). Also, where the chicks showed evidence of forgetting the learned avoidance of the yellow crumbs (Experiment 6), there was no evidence of the chicks forgetting the red crumbs (Experiment 7). This suggests that red is a more memorable signal than yellow.

In the innate avoidance experiments green tended to be treated in a similar manner to the aposematically coloured crumbs. This may be because the birds were making quick foraging decisions that did not allow careful discrimination between prey types. The chicks showed innate avoidance towards the novel aposematically coloured crumbs, and frequently appeared to treat the familiar green crumbs in the same manner. This would suggest that when novel coloured crumbs were present they were less willing to eat even familiar prey. However, the presence of additional cues appeared to enhance discrimination. Pyrazine odour enhanced discrimination between yellow and green crumbs (Experiments 1 and 4), but not red crumbs (Experiment 2). While buzzing appeared to enhance discrimination between red and green crumbs (Experiment 3), but not yellow crumbs (Experiments 3 and 4). This suggests that effect of additional signals of discrimination depends on the visual signal with which they are associated.
The chicks generalised their learned avoidance of the unpalatable yellow crumbs to the palatable green crumbs (Experiment 6); however, no such generalisation occurred between red and green crumbs (Experiment 7). Chicks generalise between similar colours such as red and orange, and yellow and orange (Ham et al. 2006). Jones et al. (2001) observed that both chicks and humans tend to generalise between similar colours, for instance yellow and green are closer to each other on the colour spectrum than red and green (Bruno and Svoronos 2005), which may explain why generalisation occurred in the former but not the latter case. If generalisation between red prey and palatable green prey is reduced then discrimination is enhanced, enhancing protection of the red aposematic insect (Guilford 1990). This suggests that an insect which is red may gain more protection from predation through innate and learned avoidance and prolonged memory of the avoidance than might a yellow insect.

As stated in Section 2.2.1 the colours used were judged to be of similar saturations. However, differences in the intensity or brightness of the visual signal may have an effect (Osorio et al. 1999), and may explain the differences between results from yellow and red. Because of this effect of intensity, it is difficult to make hard and fast predictions about the effects that warning signals will have on avian predatory behaviour. Measurement of brightness of the crumbs was outside the scope of this project. However future research would benefit from examining how different aspects of the visual signal affect the interaction with warning sounds and odours. Lindström et al. (2000) observed that pyrazine enhanced innate avoidance towards a visually conspicuous signal, experiments examining the effects of hue, brightness and patterning would provide additional interesting insight.

The robins in Experiment 8 were more willing to attack the odourless unpalatable yellow than palatable green baits, as previously suggested this may be due to the contrast of the baits with the background colour. However, this is surprising as
the same dyes were used to colour the crumbs and the pastry baits. It may be that as the dye concentration was doubled to make the baits the green baits contrasted more with the white background than the green crumbs therefore making them more aversive.

8.3 – THE MEASUREMENT OF INNATE AVOIDANCE BEHAVIOUR

The results from this thesis provide further evidence that innate avoidance behaviour is composed of at least two distinct processes. In Experiments 1 and 2 the chicks displayed dietary conservatism towards a signal to which they showed no neophobia. Chicks showed both neophobia and dietary conservatism towards red crumbs that smelt of pyrazine (Experiment 2), whereas zebra finches were noted to display dietary conservatism but not neophobia towards the same signal (Kelly and Marples 2004). This suggests that dietary conservatism is a more consistent behavioural response across species than neophobia. These findings demonstrate that the key protection that defended insects gain from innate avoidance behaviour is from predators’ dietary conservatism, and not their neophobia. Neophobia is a short-lived process and may not reflect the predator’s true feeding preferences. Dietary conservatism, on the other hand, requires a predator to have eaten several prey individuals, and thus represents a more reliable measure of the predator’s foraging decision.

Another issue to consider when measuring innate avoidance behaviour is that latency measures such as dietary conservatism appear to be more subtle and reliable measurements of innate avoidance behaviour than measurement of the number of crumbs of each colour eaten and derived measures such as eating bias (Rowe and
Guilford 1996, 1999a, b; Jetz et al. 2001). In Experiment 1 the eating bias measure did not detect differences in innate avoidance behaviour that were apparent when the latency data were examined. In Experiments 2, 3 and 4 the latency measure and the count measure reflected the similar results. However, the eating bias results from these experiments frequently suggested some non-significant trends that were not apparent from the latency measures. Only upon examination of the number of crumbs of each colour eaten, from which the eating bias was calculated, was it clear that these non-significant trends were artefacts of the calculation of the eating bias measure, and not a reflection of the chick's true behaviour (see Experiments 2 and 3 in particular). Therefore, when using derived measures such as eating bias in future research, it is essential that the results be interpreted in light of the number of crumbs of each colour attacked in order to gain an accurate picture of the behavioural responses. In every instance, avoidance behaviour detected by the measurement of the number of crumbs of each colour eaten or the eating bias was also detected by the latency measurements. This adds further weight to the suggestion that latency measures detect more subtle behavioural responses (Jetz et al. 2001), therefore making them a more reliable measurement for this type of research than count or derived measures.
The results clearly demonstrate that multimodal insect warning displays prolong innate avoidance, speed up avoidance learning and prolong memory of learned avoidance. This thesis provides the first evidence that mimics of model species that use certain multimodal warning displays need to mimic both components of the multimodal display in order gain maximal protection. The protection gained from a multimodal display is dependent on the component cues of the multimodal display. Pyrazine odour enhances innate and learned avoidance and retards forgetting of this avoidance, and reduces generalisation of the learned avoidance to palatable crumbs in close proximity, however agitated buzzing appears to have no such effect. This work provides further evidence that neophobia and dietary conservatism are two distinct processes, and makes a strong case for dietary conservatism being a more reliable measure of innate avoidance behaviour than neophobia.
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