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The Effect of the Odour of Pyrazine and Colours on Recall of Past Events and Learning in Domestic Chicks (*Gallus gallus domesticus*)

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Introduction

Until quite recently, the role played by odours as alerting signals, especially in the relationship between insectivorous bird predators and their prey, has been somewhat neglected. Attention is usually centred on warning coloration (Cott, 1940; Schuler & Roper, 1992) and behaviour, rather than smell. Bitter or sweet tastes have also, rather dubiously, been classified as alerting signals, since they can deter or encourage the final ingestion of captured insect prey or tempting fruit or seeds.

The aromatic pyrazines are exceptionally widely distributed in nature (Woolfson & Rothschild, 1990), and are one of the most successful alerting signals. They cut across the man-made classifications, utilised by both plants and vertebrate and invertebrate animals, and range from continent to continent and ocean to ocean. *Plate 2* illustrates a range of aposematic Lepidoptera and Coleoptera known to excrete the odour of pyrazine. They are not in themselves harmful or beneficial, and their message is essentially 'come' or 'go'.

The pyrazine nucleus comprises a six-membered aromatic ring containing two para-oriented tertiary nitrogen atoms. Over 100 pyrazine compounds have been identified, some of which are scentless, while the methoxyalkylpyrazines produce one of the most powerful and persistent odours known to man (Moore & Brown, 1981; Moore *et al.*, 1990). The nucleus is a stable one, which is readily derived biosynthetically from amino acids and sugar degradation products. Pyrazines are consequently widespread, not only in natural bouquets and stimulating attractive flavours, but in the decomposition or pyrolysis products of biological tissues, and by fungal decay. As odoriferous signals, they possess four especially important characteristics: (1) a low olfactory threshold (Moore *et al.*, 1990); (2) an arresting impact;

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(3) an evocative quality difficult to define, akin to the sensation of *déja vu*, which is known to be associated in man with the limbic system (Penfield & Jasper, 1954); and (4) perception at a distance, not only on contact (Guilford *et al.*, 1987).

The frequent conjugation of the odour of pyrazine with the alerting colour red was first noted in ladybird beetles (Coccinellidae) and tiger moths (Arctiidae) (Rothschild, 1961). Both characteristics are likely to be selected, since they are advantageous to both potential predator and potential insect prey, which may only become available to the former at relatively long intervals. The bird thus avoids unnecessary expenditure of energy culminating in an unpleasant experience, and the toxic insect avoids an attack which is likely to inflict injury, even if eventually futile.

Both signals occur 'right across the board' in different Orders and even Phyla; and their ability to either attract or deter is well illustrated. For example, the colour red lures birds to edible berries and fruit or nectar-laden flowers, but warns them against attacking toxic Coleoptera or Lepidoptera. The crimson wattles of turkeys can, as circumstances dictate, demonstrate either sexual attraction or masculine aggression, while baboons may charm their mates or warn rivals by displaying their bright red, swollen posteriors. We ourselves use red as a 'come' or 'go' signal, on the one hand by the coloration of children's delicious candies and, on the other, by the dye of dangerous sleeping pills. It is worth noting that the red and green cone system of primates occupies about 80 per cent of their total colour system (Rothschild & Moore, 1987).

We have assumed that the reaction of birds to so-called 'warning' colours presented in water are learned, and are not innate. The few experiments which indicate inherited aversion to certain colours in naïve chicks have been carried out with solid food and prey, not water (Schuler & Roper, 1992), and it is well known that birds may react differently to food or drink (Roper & Marples, 1997a).

In our experiments, we have tried to examine the possibility that pyrazine not only functions as a powerful alerting signal for birds, but can stimulate the vitally important recall of past events, as it is known to do for mammals (Kaye *et al.*, 1989). We are also interested in the mutual relationship between pyrazine odour and the alerting or warning colour red. Although a simple association is clearly advantageous as a means of protection, in some circumstances the relationship is synergistic and more complex, and the interactions of different colours and odours remain, at the present time, somewhat mysterious.

In a previous publication, one of us (Rothschild *et al.*, 1984) suggested that the odour of pyrazine might "assist or hasten the process of learning", and we have also attempted to test this possibility in naïve chicks.

Methods

Male domestic chicks (*Gallus gallus domesticus*), marked individually, were housed in white wooden cages (120 × 120 cm), heated, and illuminated for 12 hours light/12 hours dark. Chicks were fed *ad libitum*, and provided with plain tap water in four plastic tubes (14 cm length, 3.5 cm diameter), each attached to one of the cage walls.

Training and testing of the chicks was carried out in an experimental cage, made of a white painted, wooden waiting chamber (32 × 22 cm) connected to a testing chamber (85 × 62 cm) by a guillotine door. Both chambers opened from above. In the testing chamber, two drinking tubes were attached to each side of the wall opposite the guillotine door.

Water tubes were painted red, yellow, green, or indigo with paints manufactured by the Tambor paint factory (Israel), matched respectively to No. 45A, No. 9B, No. 133A and No. 116A on the RHS Colour Chart (Royal Horticultural Society, 1965).

Experiments involving bitter taste and pyrazine odour were carried out in a different room, to avoid contamination of the room which was used for the colour-only experiments. For bitter taste, we used quinine hydrochloride (Sigma, 0.03 M aqueous solution). The tested odour was 2-methoxy-3-isobutyl pyrazine (Pyrazine Specialities Inc., Georgia, USA), diluted by dissolving 10 μl in 100 ml distilled water. This concentration of the pyrazine solution is based on values of human olfactory thresholds (Woolfson & Rothschild, 1990). A rectangular filter paper (15 × 50 mm) was placed on each side of the water tubes. Five drops (250 μl) of odour solution or water were added to the filter papers of the test or control tubes. Odour solutions and water evaporated, and after a certain time (equivalent to about eight sessions within an experiment), filter papers were almost dry. Therefore, to ensure constant concentrations throughout the experiment, water and odour solutions were renewed after every eighth session within an experiment, by adding five drops to the filter papers.

Experimental design

Table 14.1 summarises four sets of experiments. Each set included two experiments testing effects of either colours, or colours combined with pryazine odour, on recall and learning. Each experiment consisted of five or six replicates with 10–13 chicks in each. Only chicks which completed the training process (see below for details) were included in the analysis. Ninety-one per cent of the total number of chicks which were used successfully completed the learning process and, accordingly, the total number of chicks in each experiment ranged between 40 and 61. Overall, 393 chicks were tested and the results analysed.

Our previous experience with chicks has shown that a total of 30 chicks is a manageable number to handle at one time, as it allows completion of all learning sessions of DAYS 5 and 6 (see below) within a reasonable time. If experiments involve more chicks, the learning process lasts too long, and chicks do not co-operate as they do at the beginning of the day. Each replicate consisted of about 10 chicks, and therefore three replicates were tested each time. The three replicates were randomly chosen from all eight experiments presented in *Table 14.1*, so that sets of experiments were broken down and, as a result, different replicates of the same experiment were tested at different times. This design obtained internal validity (Keppel, 1982, p. 339)

Table 14.1. Experiments designed to investigate the effect of pyrazine odour and colours (red, yellow, green, and indigo) on recall and learning (see text).

Experimental set	Colours with/without pyrazine odour (no. replicates; total no. chicks tested)	
1	red (5;47)	red + pyrazine (6;54)
2	yellow (6;44)	yellow + pyrazine (5;43)
3	green (5;50)	green + pyrazine (5;49)
4	indigo (5;45)	indigo + pyrazine (6;61)

and allowed inter-experiment comparisons, as it took into consideration the arbitrariness of the assays, which might be due to the use of different hatchlings, different times of the year, and other unknown factors between the runs.

The general frame of the experiments

DAYS 1–3 Day-old chicks were kept in housing cages where food and water were available *ad libitum*. Water was provided in five water tubes, each of a different colour (red, yellow, green, indigo, and white), with a daily random change in their location.

DAY 4 After overnight water deprivation, chicks were individually trained to drink from tubes in the experimental cage. Each chick was given five training sessions, in random order. During each session, the chick stayed in the waiting chamber for 15 seconds, and was then moved through the guillotine door into the testing chamber, which had two water tubes placed on the far wall, coloured alike, and filled with tap water (referred to as 'neutral colour'). The chick was allowed 30 seconds to drink from either tube before being returned to the housing cage.

DAYS 5 and 6 After overnight water deprivation, the chicks were taught the association between:

(1) a specific colour and taste of water,
(2) a colour, pyrazine odour, and taste of water.

One of the water tubes was permanently white, containing plain tap water (referred to as the 'white tube'). The other tube contained bitter water, coloured in one of the tested colours, either with or without pyrazine odour (referred to as the 'test tube'). On DAY 5, each chick had five learning sessions similar to the ones on DAY 4, but now it was given the choice of drinking either from the white or test tube. The location of the tubes was changed randomly after every session. From the end of DAY 5, chicks were given water *ad libitum* in neutral coloured tubes in the housing cage until the end of the experiment on DAY 21.

On DAY 6, after overnight water deprivation, each chick was given as many sessions as needed to reach the 'learning criterion', defined as drinking from the white tube (the 'correct' one) on three consecutive sessions. Chicks which only drank from the white tube in all sessions were removed from the experiment.

DAYS 7–10 Chicks were given water *ad libitum* in the housing cage.

DAY 11 After overnight water deprivation, chicks were first tested for recall. They were randomly chosen and individually introduced to the experimental cage, where they were exposed to the same situation as the one presented to them on DAYS 5 and 6, except that now both tubes (white and test) were filled with plain tap water. This testing regime resembled the natural situation, in which a predator re-encounters a specific combination of visual and odoriferous signals already known to be disagreeable. Each chick had only one session (described above) and had to choose between the two tubes. Chicks that drank from the white tube (the 'correct' one, originally containing plain tap water) were considered to recall the association with colour and bitter water correctly, and were put back into the housing cage for future recalls.

Chicks which drank from the test tube (the 'wrong' one, which originally contained bitter water) were removed from the experiment. Length of recall was therefore defined as the number of days from the learning day during which the chick remembered to avoid the tested tube.

DAYS 13, 14, 15, 18 and 21 The same recall procedure was carried out as on DAY 11. The location of tubes in the experimental cage was randomly changed between recalls. The experiment ended on DAY 21.

Data analysis

To test the effect of different colours and pyrazine odour, we used two-way Analysis of Variance. Survival analysis (SAS, 1990) was used to test the effect of different colours and pyrazine odour on recall. We used the LIFETEST procedure, which tests for equality of survival curves across strata (Cox & Oakes, 1984), for survival analysis.

Results

EFFECTS OF COLOURS (WITHOUT PYRAZINE ODOUR) ON RECALL

Figure 14.1 presents recall curves when bitter water was associated with coloured tubes (red, yellow, green, and indigo). Statistical analysis showed that three colours (red, yellow, and green), out of the four tested, did not differ significantly in their effect on recall. Indigo was the only colour which differed significantly, by improving recall (comparison of indigo v. red ($\chi^2 = 4.6093$, df = 1, $P = 0.031$); indigo v. yellow ($\chi^2 = 14.7308$, df = 1, $P = 0.0001$); indigo v. green ($\chi^2 = 4.5870$, df = 1, $P = 0.0322$)). The trend (from best to worst recall) seen in *Figure 14.1* is: indigo, green, red, and yellow.

EFFECTS OF PYRAZINE ODOUR ON RECALL

Figure 14.2 presents recall curves when bitter taste was associated with the colour red, yellow, green, and indigo, with or without the odour of pyrazine. The results of statistical analyses are presented in *Table 14.2*, and show that addition of pyrazine odour significantly improved recall of bitter taste associated with yellow, green, and red tubes. Indigo was the only coloured tube to which the addition of pyrazine odour did not improve recall.

THE EFFECT OF COLOURS AND PYRAZINE ODOUR ON LEARNING

Table 14.3 summarises the results on the effect of colours, with or without pyrazine odour, on the learning process. Statistical analysis of these data showed that:

(1) there was no significant difference between the mean number of sessions required to reach the learning criterion when the bitter taste of water was associated with different colours;

(2) the addition of pyrazine odour to the association between bitter taste of water and

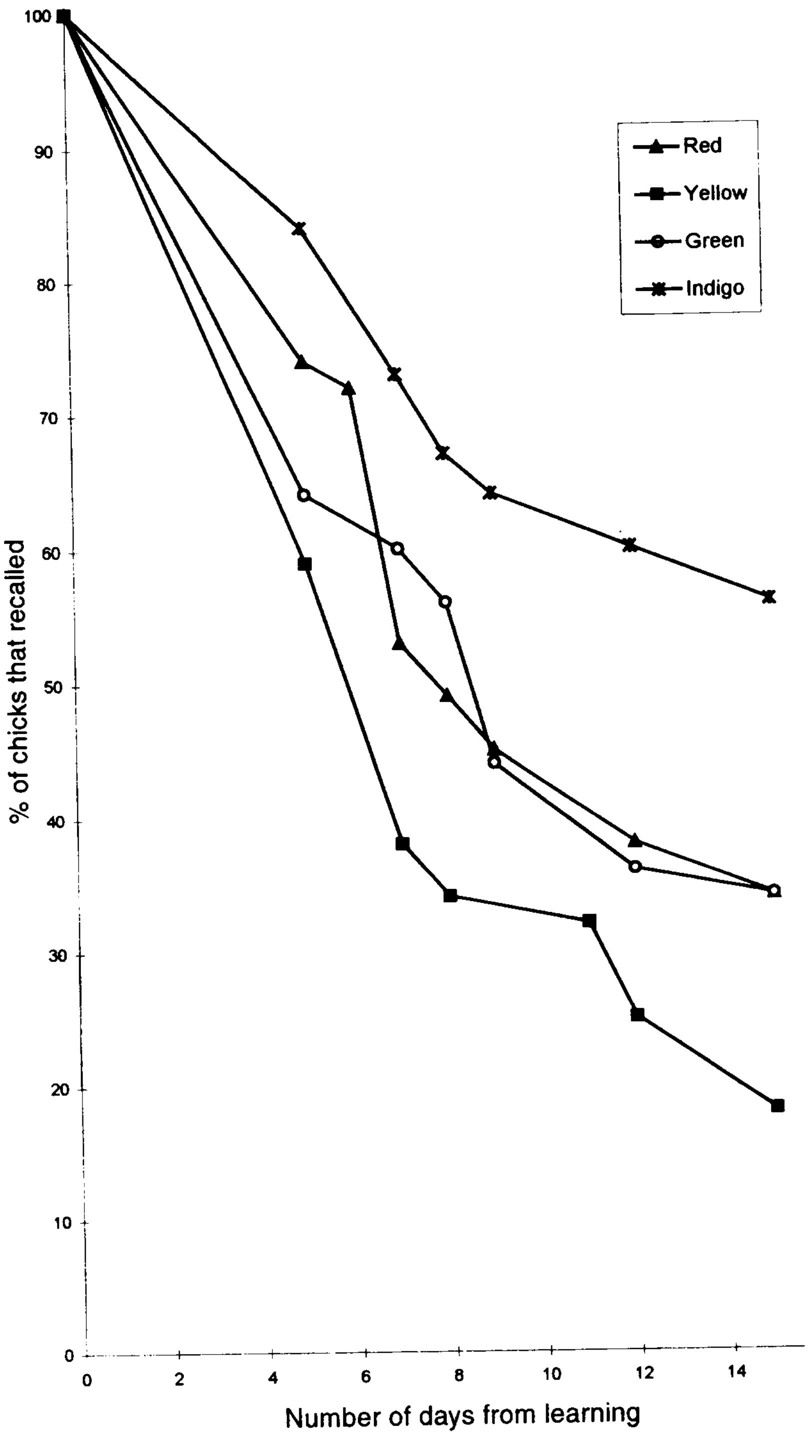

Figure 14.1. Percentage of chicks recalling association between bitter water and colour (▲, red; ■, yellow; ○ green; ✖, indigo) of the water tube, as a function of the number of days from learning.

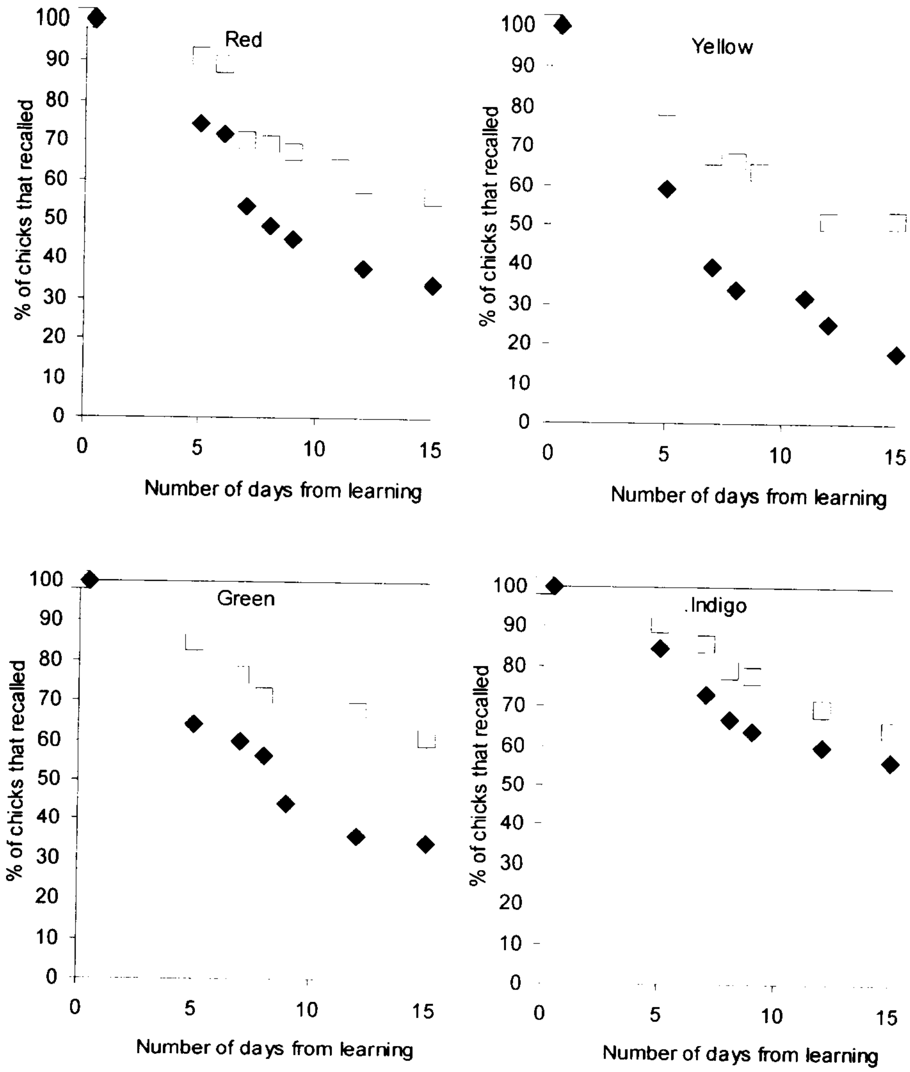

Figure 14.2. Percentage of chicks that recalled association between bitter water and the colour of the water tube, with (□) or without (◆) pyrazine odour, as a function of the number of days from learning.

Table 14.2. Chi-square values for comparisons between different recall curves (see *Figure 14.2*).

Colour of water tube associated with the bitter taste	$\chi^2_{df=1}$ for comparison of the recall curves for this association and the same with pyrazine odour
red	5.54 ($P = 0.019$)
yellow	10.84 ($P = 0.001$)
green	7.68 ($P = 0.006$)
indigo	not significant

Table 14.3. Mean (± s.e.) to reach the learning criterion (see text) for an association between bitter taste and a colour with/without pyrazine odour.

Colour	Without odour	With pyrazine odour
red	10.0 ± 0.29 (n = 47)	8.8 ± 0.17 (n = 54)
yellow	9.0 ± 0.21 (n = 44)	8.6 ± 0.15 (n = 43)
green	9.7 ± 0.26 (n = 50)	9.0 ± 0.20 (n = 49)
indigo	9.7 ± 0.28 (n = 45)	8.8 ± 0.15 (n = 61)

the colour of the water tube always shortened the learning process, and fewer sessions were then required to reach the learning criterion (*Table 14.3*). Overall, the odour effect was found to be significant (F = 26.95, df = 1,3, $P = 0.0001$). In order to carry out multiple comparison tests for the different colour–odour combinations and to control for experimental error, we employed Sidak's multiplicative inequality procedure (Sidak, 1967). Accordingly, when α is set to 0.05 and four comparisons are made, α' for each single comparison equals 0.0127. Results of these tests showed that the effect varied as follows: when bitter water was associated with red or indigo tubes, the addition of pyrazine odour shortened the learning process significantly (F = 10.10, df = 1,104, $P = 0.002$ and F = 11.50, df = 1,99, $P = 0.001$ respectively); when bitter water was associated with green and yellow tubes, the tested odour had no significant effect on the learning process (F = 4.45, df = 1,97, $P = 0.038$ and F = 2.23, df = 1,85, $P = 0.139$ respectively).

Discussion

The chemical defences of insects are amazingly varied (Blum, 1981) but, on the whole, alerting signals are comparatively limited. This is particularly evident in the case of pyrazine odour, which remains dominant and assertive, even when the accompanying visual alerting colours are arranged in completely different patterns. Thus, for example among the tiger moths (Arctiidae), the white ermine moth (*Spilosoma lubricipida*) is snow white, with a few black speckles and a bright yellow abdomen, while the cinnabar moth (*Tyria jacobaeae*) is scarlet, with blue-black forewings, and the cream spot tiger moth (*Arctia villica*) has bright yellow hindwings with black spots, black forewings with large pale yellow blotches, and a scarlet abdomen. Yet all three excrete odoriferous pyrazines (Rothschild, 1961; Moore *et al.*, 1990) (*Plate 2*).

Species of the beetle genus *Pseudolycus* (Oedemeridae) are described by Moore & Brown (1989) as an example of sophisticated mimicry since "they have developed different colour patterns over their extensive ranges ... each to match a particular sympatric model and all smell of methoxy-alkyl pyrazine."

In our laboratory conditions, odours overshadowed or eclipsed colour cues when chicks experienced them together. Our trials with coloured drinking tubes and the odour of pyrazine show this tendency where recall of bitter water is concerned. It was only when pyrazine odour was added to the coloured tubes that recall was significantly improved.

Marples & Roper (1996) found that the odour of almond oil shortened avoidance learning in chicks, thus drawing attention to another effective avian alerting odour. However, they do not state whether or not HCN, which occurs naturally in the almond oil, had been extracted. HCN has been recorded from over a thousand species of plants (Jones, 1979), and it may well rival pyrazine as a common botanical alerting signal. In burnet moths (*Zygaena* spp.), HCN functions as both an alerting and warning signal combined. Curiously enough, burnet moths also excrete pyrazine (Rothschild, 1985; Moore *et al.*, 1990), and they are avoided by almost all bird predators.

Like Roper & Marples (1997b), we also found that coloured drinking tubes were ineffectual in shortening the learning process for quinine-tainted water; but when we offered them to chicks, together with the pyrazine odour, only two colour–odour combinations (red and pyrazine, and indigo and pyrazine) yielded a significant reduction in learning time. Thus, red and pyrazine was the only association which significantly improved both recall and learning of bitter taste. This association is not unexpected from an evolutionary standpoint. Pyrazine (see earlier) has so many features which qualify it for the role of a superior alerting signal, not least of which is its availability. Red as an alerting colour *par excellence,* one assumes, must have evolved in response to the need of a conspicuous colour "in a green world beneath a blue vault". Furthermore, Varela *et al.* (1993) pointed out that, in the various species of birds they examined, the pigment (iodopsin) absorbing at the longest wavelength near 570 mm is by far the most abundant. It appears to be the visual pigment that "dominates phototopic spectral sensitivity of the birds". The combination of pyrazines and the colour red as an alerting signal is obviously felicitous.

We have failed to find an explanation for the results of some of our experiments: thus, the chicks' reaction to the indigo coloured tube is baffling. It proved to be the only coloured tube which stimulated recall without the addition of pyrazine and yet, when associated with that odour, unlike the other coloured tubes, it did not improve recall of bitter water. Nevertheless, it elicited shortening of the learning process.

The indigo tube was very dark in shade and tone, considerably darker than the other tubes involved (see earlier), and black paint was certainly mixed most lavishly with indigo to obtain the blue-black coloration characteristic of many aposematic insects. This tube contrasted sharply with the all-white walls of the experimental cage, and possibly we may have unwittingly introduced the potent element of contrast, which affected the chicks' reaction to colours and odours. Authors writing at the turn of the last century (Marshall, 1902; Swynnerton, 1919) noted that ground-foraging birds avoided toxic ground-dwelling beetles which adopted all-black as a warning signal, and exposed themselves against a light background of sandy soil.

A somewhat different puzzle concerns Rowe & Guilford's (1996) finding that pyrazine odour triggered aversion in chicks to food coloured red or yellow, but not green. We could find no support for this observation in our experiments. The discrepancy may solely be due to their use of solids, rather than fluids. It is well known, as we have indicated, that birds react differently to food or drink. Gillette *et al.* (1980) showed that colour aversion in domestic chicks is not easily formed when the colour is associated with fluids, rather than solids. One of us (M. Rothschild, *unpublished*) found that many adult quails developed a permanent aversion to red-coloured water tainted with quinine, but relished various solid food to which it had been added in a visible surface layer.

Great caution is necessary when attempting to interpret such experiments, since bird behaviour is often changeable and capricious, varying from one individual to another, or from brood to brood, and furthermore, bird colour vision is not well understood. Varela *et al.* (1993) pointed out "the tetra or pentachromatic colour space of birds appears to be the most complex in nature, and is likely involved in virtually all areas of the animals' lives", but the authors refer to the different and mysterious functions, the solution of which presents an "appealing facet of natural history".

Nature is, of necessity, parsimonious, opportunistic, and immensely variable. It is, however, suprising for us to find birds, which have hitherto been considered relatively indifferent to smell and dominated by colour cues or the behaviour of potential insect prey, responding so decisively to odours.

Although, at first sight, alerting signals may appear to convey unequivocal messages of 'come' or 'go', on closer examination, they are seen to be more complex. In many plants, and in the melon fly (*Dacus cucurbitae*) (Baker *et al.*, 1982), they convey different messages simultaneously, attracting one species and repelling another. Again, the same signals, used at long intervals, can convey opposite meanings as circumstances dictate. A display of red feathers may be employed to scare off rivals, or attract prospective mates. Moreover, the same message is often conveyed by simultaneous appeal to visual, auditory, or olfactory sensibilities of predators. In mammals, the odour of pyrazines can delay sexual development (Jemiolo & Novotny, 1994), as well as stimulate recall (Kaye *et al.*, 1989), or suppress the development of T cells in mice (Woolfson & Rothschild, 1990). In ants, pyrazine plays a variety of different roles, functioning, for instance, as a trail-laying substance, a defensive spray, or escape pheromone (Brophy, 1989), and in these cases colours are not involved.

It would come as no suprise to find that the type of alerting signal we have investigated is the 'tip of the iceberg', and that pyrazine and many other aromatic substances, with or without a specific colour or sound association, will be found to play a significant role in animal neurophysiology.

Acknowledgements

We thank Yoram Yom-Tov for his support, Yael Alberton for statistical advice and analysis, and Dina Lipkind for technical help. We are also grateful for useful discussions with Robin Aplin, Gunnar Bergström, and Charles Lane. The work was supported by the Ministry of Science, Israel.

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