Animal behaviour

Fruit flies learn to avoid odours associated with virulent infection

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While learning to avoid toxic food is common in mammals and occurs in some insects, learning to avoid cues associated with infectious pathogens has received little attention. We demonstrate that Drosophila melanogaster show olfactory learning in response to infection with their virulent intestinal pathogen Pseudomonas entomophila. This pathogen was not aversive to taste when added to food. Nonetheless, flies exposed for 3 h to food laced with P. entomophila, and scented with an odorant, became subsequently less likely to choose this odorant than flies exposed to pathogen-laced food scented with another odorant. No such effect occurred after an otherwise identical treatment with an avirulent mutant of P. entomophila, indicating that the response is mediated by pathogen virulence. These results demonstrate that a virulent pathogen infection can act as an aversive unconditioned stimulus which flies can associate with food odours, and thus become less attracted to pathogen-contaminated food.

1. Introduction

Pathogen avoidance has obvious adaptive advantages. However, the presence of pathogens in the environment is often not directly detectable, nor stably associated with sensory cues that would permit the evolution of genetically determined, innate avoidance. In such cases, learning to avoid cues associated with disease symptoms becomes important as a way of reducing subsequent exposure to pathogens. Avoidance of food previously associated with malaise is well studied in generalist mammal species such as rats and mice [1,2]. Analogous studies in insects are scarce, but some insects also learn to avoid food associated with taste-independent physiological effects of toxic compounds [3,4] (although others apparently do not [5]). However, all these studies relied on malaise induced by ingestion or injection of toxic compounds, such as lithium chloride, alkaloids or glycosides, or immune elicitors, for example lipopolysaccharides. Yet, the perception of physiological effects of these compounds may be qualitatively or quantitatively different from perception of a pathogenic infection. Furthermore, an infection may compromise learning ability [6,7]. That natural pathogen infection can act as an aversive stimulus mediating learned pathogen avoidance has, to our knowledge, only been directly demonstrated in the nematode Caenorhabditis elegans, which learns to avoid odours emitted by pathogenic bacteria [8].

In this study, we test whether Drosophila melanogaster flies learn to avoid arbitrary olfactory cues linked to infection with their natural virulent intestinal pathogen Pseudomonas entomophila [9]. After first showing that this pathogen is not aversive to taste, we test whether exposure to P. entomophila-laced food scented with an odor affect subsequent choice between this food and food scented with an alternative odor. To separate the effect owing to pathogen virulence from other consequences of pathogen exposure, we use a natural virulent pathogen strain (‘virulent strain’) as well as an otherwise genetically identical avirulent mutant carrying a mutation in the virulence regulator GacA (‘harmless strain’).
2. Material and methods

(a) Design of experiments

The data were obtained by recording groups of 40–50 flies choosing between two food substrates in small transparent cages. The origins of flies and bacteria and the experimental procedures are detailed in the electronic supplementary material.

We first used this approach to test whether the bacteria-laced food is initially appetitive or aversive to flies never previously exposed to the pathogen (experiment 1). Flies were given the choice between a food substrate inoculated with bacteria (virulent or harmless) and a bacteria-free food substrate; they were recorded for 2 h (N = 8 cages per strain).

In the main experiment (experiment 2), we tested whether an association of a food odour with *P. entomophila* affects subsequent choice between this and an alternative odour, and whether this depends on the bacteria being virulent. Following a standard approach to *Drosophila* olfactory learning [10], we assessed learning by conditioning groups of flies with different odours and comparing their choice between these odours. Amyl acetate (AA) and ethyl hexanoate (EH) were used as odors; these esters occur in decomposing fruit. The assay consisted of two phases. During the conditioning phase (3 h), flies received a single food substrate inoculated with bacteria (virulent or harmless) and supplemented with one of the two odorants as the conditioned stimulus. We thus used single-odour conditioning. (Two-odor discrimination conditioning was not used because it would have been difficult for the flies to discern which odour was the origin of the malaise [4]). The conditioning phase was immediately followed by the test phase (2 h), in which the flies could choose between two food substrates, one scented with AA and one with EH (N = 30–33 cages per odorant and strain). To minimize differences in the olfactory and gustatory context between conditioning and test phase, both substrates used in the test phase were inoculated with the harmless strain.

(b) Statistical analysis

Raw data for both experiments are available in the electronic supplementary material. The analysis focused on the relative preference for the two substrates, defined as the number of flies present on one versus the other substrate. These numbers were obtained from snapshots, taken at 15 min intervals, starting at 30 min after providing the substrates; flies located elsewhere in the cage provide no information about food preference and so were excluded from the analysis (see the electronic supplementary material). Data were analysed with a generalized mixed model (procedure GLIMMIX of SAS v. 9.3), assuming a binomial error distribution and logit link. In experiment 1, preference for food inoculated with *P. entomophila* (virulent or harmless strain) was the response variable; the conditioned odour, the strain used in conditioning and their interaction were the effects (the 'learning index' as defined in Drosophila literature [10]) was 0.232 (s.e. 0.080) and the treatment effects were consistent (figure 1; for both strains *p* = 0.012). The estimated magnitude of this effect (the 'learning index' as defined in Drosophila literature [10]) was 0.232 (s.e. 0.084). By contrast, the likelihood of flies exposed to the virulent strain paired with EH were subsequently more likely to choose AA-scented food than flies similarly conditioned with the virulent pathogen and AA (figure 2; separate analysis for the virulent strain: χ² = 6.4, *p* = 0.012). The estimated magnitude of this effect (the 'learning index' as defined in Drosophila literature [10]) was 0.232 (s.e. 0.084).

In experiment 2, how the identity of the odorant associated with pathogens affected subsequent odorant preference depended on the pathogen strain (conditioned odour × strain χ² = 5.3, *p* = 0.022). Flies exposed to the virulent strain paired with EH were subsequently more likely to choose AA-scented food than flies similarly conditioned with the virulent pathogen and AA (figure 2; separate analysis for the virulent strain: χ² = 6.4, *p* = 0.012). The estimated magnitude of this effect (the 'learning index' as defined in Drosophila literature [10]) was 0.232 (s.e. 0.084). By contrast, the likelihood of choosing AA versus EH was unaffected by prior association between odorants and the harmless strain (figure 2; separate analysis for the harmless strain: χ² = 0.4, *p* = 0.51). The relative preference for AA versus EH did not change over time (χ² = 3.1, *p* = 0.080) and the treatment effects were consistent (figure 2a; *p* > 0.4 for all interactions between treatments and time). The motivation to feed in the test phase was higher for flies conditioned with the virulent than the harmless strain, but additional analysis excluded this as a potential confounding effect for the results reported above (electronic supplementary material, figure S2b).

3. Results

In experiment 1, irrespective of the bacterial strain, naive flies preferred bacteria-laced over standard food substrate (figure 1; for both strains t₄₄ > 3.8, *p* < 0.002; one-sample *t*-test on least-square logit means). Preference was not affected by bacterial strain (χ² = 0.01, *p* = 0.91), nor changed systematically with time (time χ² = 2.7, *p* = 0.10; strain × time interaction χ² = 0.6, *p* = 0.45). The motivation to feed (i.e. the proportion of flies located on food substrates versus elsewhere in the cage) was not affected by the bacterial strain (electronic supplementary material, figure S2a).

In experiment 2, how the identity of the odorant associated with pathogens affected subsequent odorant preference depended on the pathogen strain (conditioned odour × strain χ² = 5.3, *p* = 0.022). Flies exposed to the virulent strain paired with EH were subsequently more likely to choose AA-scented food than flies similarly conditioned with the virulent pathogen and AA (figure 2; separate analysis for the virulent strain: χ² = 6.4, *p* = 0.012). The estimated magnitude of this effect (the ‘learning index’ as defined in *Drosophila* literature [10]) was 0.232 (s.e. = 0.084). By contrast, the likelihood of choosing AA versus EH was unaffected by prior association between odorants and the harmless strain (figure 2; separate analysis for the harmless strain: χ² = 0.4, *p* = 0.51). The relative preference for AA versus EH did not change over time (χ² = 3.1, *p* = 0.080) and the treatment effects were consistent (figure 2a; *p* > 0.4 for all interactions between treatments and time). The motivation to feed in the test phase was higher for flies conditioned with the virulent than the harmless strain, but additional analysis excluded this as a potential confounding effect for the results reported above (electronic supplementary material, figure S2b).

4. Discussion

Rather than showing unconditional avoidance, flies never previously exposed to the pathogen showed a preference for food laced with *P. entomophila* over pathogen-free food. This attraction was independent of whether the virulent or
the harmless pathogen strain was used. It presumably reflects odour- and/or taste-based attraction to the bacteria themselves, products of their metabolic activity or traces of bacterial broth. This seemingly maladaptive behaviour is not surprising, given that *Drosophila* normally feed on microbe-colonized substrates. Furthermore, the pathogen originates from the Caribbean [9] and is thus likely to be evolutionarily novel to our fly population. Similar initial attraction to pathogens has been reported in *C. elegans* [8].

Despite the flavour of bacteria being attractive to naïve flies, flies which experienced an odour paired with the virulent pathogen were subsequently less likely to choose food scented with this odour than flies conditioned with the alternative odour–virulent pathogen pairing. That this did not occur when the odour was paired with the avirulent pathogen strain indicates that it is not a consequence of simple exposure to the odours, mediated by processes such as habituation [11]. Furthermore, the observed attraction to bacteria-laced food excludes associative learning based on the pathogen’s flavour acting as an aversive unconditioned stimulus. Finally, even though intestinal infection with the virulent pathogen strain leads to high mortality, under our infection protocol, this mortality does not commence before 16 h from the onset of pathogen exposure (A.B. 2012, unpublished results); virtually no mortality was observed during the duration of assays reported here. Thus, our results cannot be explained by a sampling bias caused by differential mortality.

The most parsimonious explanation for the difference in preference is flies learning to associate the odorant with the virulent pathogen and the negative physiological consequences of infection with *P. entomophila*. Three hours during which flies were exposed to the odour–pathogen association are sufficient for the pathogen to start damaging the gut lining and inducing immune response [12]. Crucially, the harmless strain induces neither but is otherwise genetically identical to the virulent strain [9]. Thus, the absence of response to association between odour and the harmless strain indicates that the responses to association with the virulent strain are mediated by the pathogen-inflicted damage or by the fly’s immune response. The degree to which odour preference was affected by previous association with pathogens, quantified as a learning index of 0.23, was similar in magnitude to the effect of association of odours with unpalatable taste [13–15]. Even such a modest reduction of exposure to pathogens associated with attractive food sources could have important consequences for Darwinian fitness, not only through reduction in mortality, but also through redirecting oviposition away from infectious substrates. However, the adaptive value of malaise-based associative learning would be limited by the fact that the perception of malaise does not change immediately upon switching from a pathogen-contaminated to pathogen-free food. It would thus be impossible to learn to discriminate between pathogen-contaminated and pathogen-free foods encountered in quick succession [4,16]. Therefore, one would expect that the insect would learn to avoid all food-related cues encountered while the symptoms of infection are developing [4,16], possibly promoting preference for novel food sources.

This study demonstrates that learned pathogen avoidance is possible in *Drosophila* (and thus presumably in other insects) because pathogen infection can indeed act as an aversive unconditioned stimulus in olfactory learning. A similar phenomenon has been shown in *C. elegans* [8]. However, the *C. elegans* study demonstrated learned avoidance of volatiles produced by the pathogen itself, we show that this process can reduce attraction to food contaminated with pathogens even if the pathogens have no smell or taste.

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### References


