

Fruit flies learn to avoid odours associated with virulent infection

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Summary

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 hours 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 such as 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 *C. elegans*, which learns to avoid odours emitted by pathogenic bacteria [8].

In this study we test if *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 if exposure to *P. entomophila*-laced food scented with an odour affects subsequent choice between this food and food scented with an alternative odour. To separate the effect due 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 Electronic Supplementary Material (ESM).

We first used this approach to test if 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 if 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 odorants; 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-odour 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 as an electronic supplement. 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 ESM). 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 the bacteria-laced versus bacteria-free substrate was the response variable and bacterial strain was the treatment. In experiment 2, the preference for the AA-versus EH-supplemented substrate in the test phase was the response variable; the conditioned odour, the strain used in conditioning and their interaction were the treatments. We also performed a

separate analysis for each pathogen treatment. All analyses included cage as a random subject nested within treatments, time as a continuous variable, and an overdispersion parameter. This model thus accounts for the non-independence of individual flies sharing a cage and of consecutive time points from the same cage. Interactions between treatments and time were tested and removed from the model if not significant. To plot the results, the mean proportions and their standard errors at each time point were estimated with a model analogous to those described above but applied separately to each treatment and treating time as a categorical variable. We also analysed the pooled number of flies on both food substrates versus elsewhere in the cage as a measure of motivation to feed (see ESM).

3. Results

In experiment 1, irrespective of the bacterial strain, naïve flies preferred bacteria-laced over standard food substrate (figure 1; for both strains $t_{14} > 3.8$, $P < 0.002$; one-sample t -test on least-square logit means). Preference was not affected by bacterial strain ($\chi^2_1 = 0.01$, $P = 0.91$), nor changed systematically with time (time $\chi^2_1 = 2.7$, $P = 0.10$; strain \times time interaction $\chi^2_1 = 0.6$, $P = 0.45$). 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 (supplementary 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 \times strain $\chi^2_1 = 5.3$, $P = 0.022$). 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: $\chi^2_1 = 6.4$, $P = 0.012$). The estimated magnitude of this effect (the "learning index" as defined in *Drosophila* literature [10]) was 0.232 (SE = 0.084). In 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: $\chi^2_1 = 0.4$, $P = 0.51$). The relative preference for AA versus EH did not change over time ($\chi^2_1 = 3.1$, $P = 0.080$) 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 (supplementary 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 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. Babin, 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 negative physiological consequences of infection with *P. entomophila*. The 3 h 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 a quick succession [4,16]. Therefore, one would expect that the animal 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, while 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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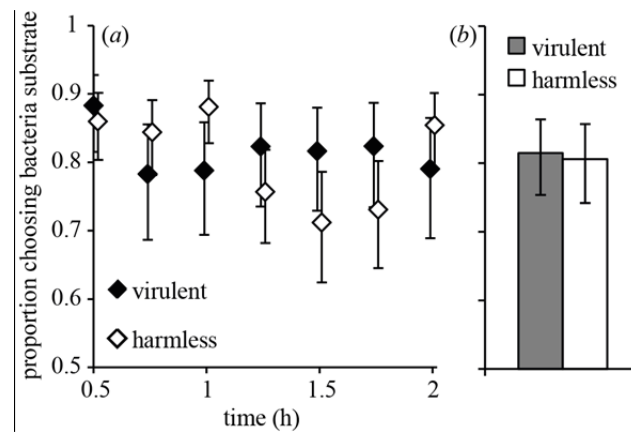


Figure 1. Preference for food inoculated with *P. entomophila* (virulent or harmless strain) versus bacteria-free food (experiment 1). (a) Estimated mean proportions choosing bacteria-laced food at each time point relative to flies present on both food substrates. (b) Least-square (marginal) means (\pm SE) estimated in generalized mixed model. $N = 8$ cages per strain. The data for all strains were collected at the same time points; the symbols in panel a (and in figure 2a) have been slightly offset for better readability.

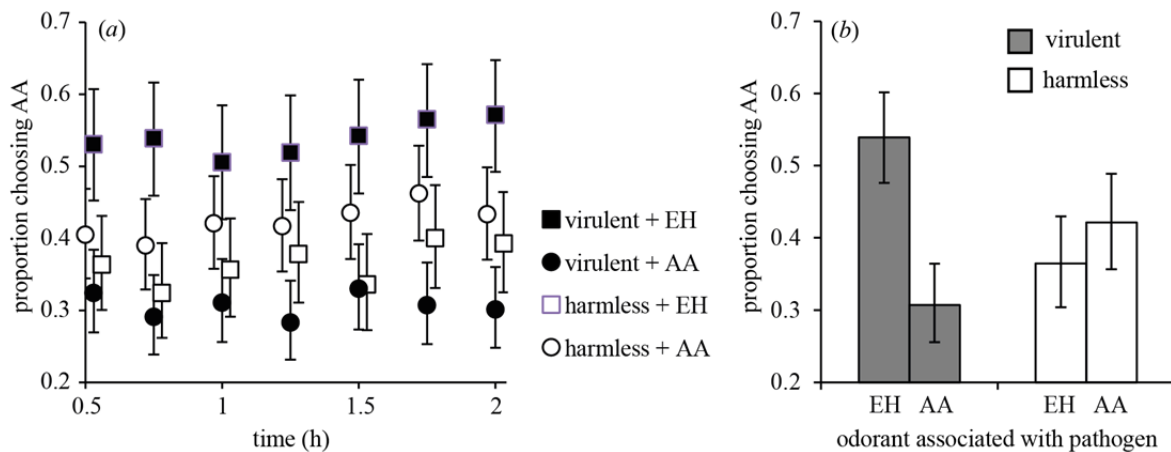


Figure 2. The effect of association between an odorant (EH or AA) and pathogen (virulent or harmless) on subsequent choice between food substrates scented with AA and EH (experiment 2). (a) Estimated mean proportions (\pm SE) choosing AA-scented food at each time point. (b) Least-square treatment means estimated in generalized mixed model. $N = 30$ -33 cages per odorant-strain combination.

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Electronic Supplementary Material:

- Detailed methods and analysis with figure S1
- Supplementary results with figure S2

Detailed methods and analysis

Flies and pathogens

We used an outbred laboratory population of *Drosophila melanogaster*, derived from over 200 fertile females collected in Valais (Switzerland) and maintained in the laboratory at the size of several hundred breeding individuals for four years before the onset of this study. Flies were raised at 25 °C on a standard cornmeal-yeast food medium under controlled larval density. For experiments, 2-6 days old females were collected under CO₂ anaesthesia, allowed to recover for 24-30 h on fresh food, and subsequently starved for 2 hours in empty vials in order to increase their motivation to feed. As females were housed with males for the first two days of their adult life, almost of all them would have mated. Each fly was only used once.

The virulent generalist entomopathogen *Pseudomonas entomophila* (“virulent strain”) and the avirulent *gacA* mutant derived from the virulent strain (“harmless strain”) were kindly provided by Bruno Lemaitre (EPFL, Lausanne, Switzerland). The virulent strain was originally isolated from a female *D. melanogaster* in Guadeloupe (France) [1]. Despite eliciting a rapid and strong immune response, ingested virulent *P. entomophila* multiply in the gut, cause gut damage and, at least at high doses used in infection experiments and upon sustained exposure (over 20 h), result in mortality of 40 - 85 % within 1-4 days, with some evidence for the effect being dose-dependent ([1,2]).

Following reference [1], bacteria were grown at 28°C in standard LB broth (5 g Bactoyeast®, 10 g Bactotryptone® and 10 g NaCl per litre of distilled water). A pellet was collected for each bacterial strain after culture centrifugation (3000 rpm, 20 min, 4 °C). Pellet optical density at 600 nm was adjusted with sterile 0.9 % saline buffer to 200 ($\approx 10^{11}$ cells/ml).

Behavioural recordings

The data were obtained by recording flies given a choice between two food substrates. The assays were performed in transparent polystyrene cages (102 × 83 × 40 mm), with symmetrical openings situated diagonally at the bottom, to which two externally removable Petri dishes (Ø 35 mm) could be attached (figure S1). The Petri dishes were filled with 10 ml of the standard yeast-cornmeal-sugar food substrate. According to the treatment (as described in the main text), the food dishes were scented with an odorant, amyl acetate (AA) or ethyl hexanoate (EH) and/or supplemented with the virulent or the

harmless bacteria. To scent the food, the odorant was first diluted to 1% concentration in 1,2-propanediol and then 1 ml of this solution was added per 100 ml of food substrate. To apply bacteria, 50 μl of bacterial suspension describe above ($\approx 5 \times 10^9$ bacterial cells) was spread uniformly across the surface of the medium after it cooled.

Forty-fifty females were transferred to each cage; cages were placed in recording boxes uniformly illuminated with white LEDs; the orientation of the cages within the recording boxes was randomized. The data were obtained by counting flies present on each food substrate on snapshots taken with a webcam (Logitech) at 15 min intervals, starting at 30 min after the flies were introduced in the cage to allow them to settle down after handling.

The experiments were spread over several months. Experiment 1 was carried out in two blocks each with four replicates of each treatment. Experiment 2 was arranged in 33 blocks consisting of one replicate of each of the four treatments assayed in parallel. A random block effect was initially included in the analysis, but the corresponding variance component was zero for both experiments, and including it had a negligible effect on tests of the fixed effects. We therefore removed it from the final analysis reported in the paper.

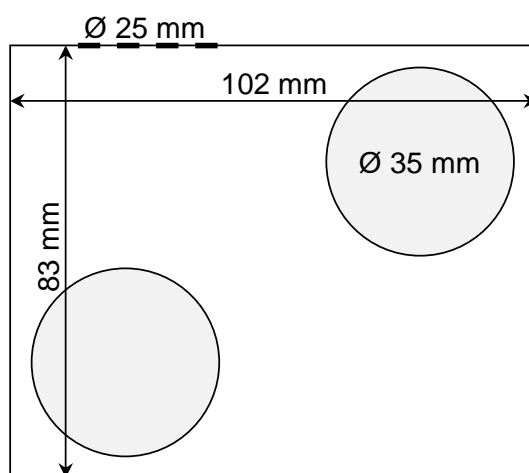


Figure S1. The design of transparent polystyrene cages used for the behavioural recordings. Two replaceable Petri dishes (grey) are attached diagonally at the bottom of the cage for food substrates; the dashed lines indicate the position of mesh-covered ventilation holes (diameter 25 mm) on the walls of the cage. Cage height was 40 mm.

Supplementary Results

Proportion of flies on food substrates versus elsewhere in cage

We present here the results concerning the proportion of flies that were recorded on both food substrates versus flies located elsewhere in the cage. Following the standard approach [3], the latter group was excluded from the analysis of relative food preference reported in the main text. The proportion of flies on versus off food substrates presumably reflects the motivation to feed and oviposit; for the sake of brevity we refer to it as the proportion feeding (keeping in mind that flies present on food must necessarily be feeding all the time). We employed the same statistical models as for the food preference in the main text, except that a quadratic term in time was added to the analysis of experiment 2 to account for the strongly non-linear response.

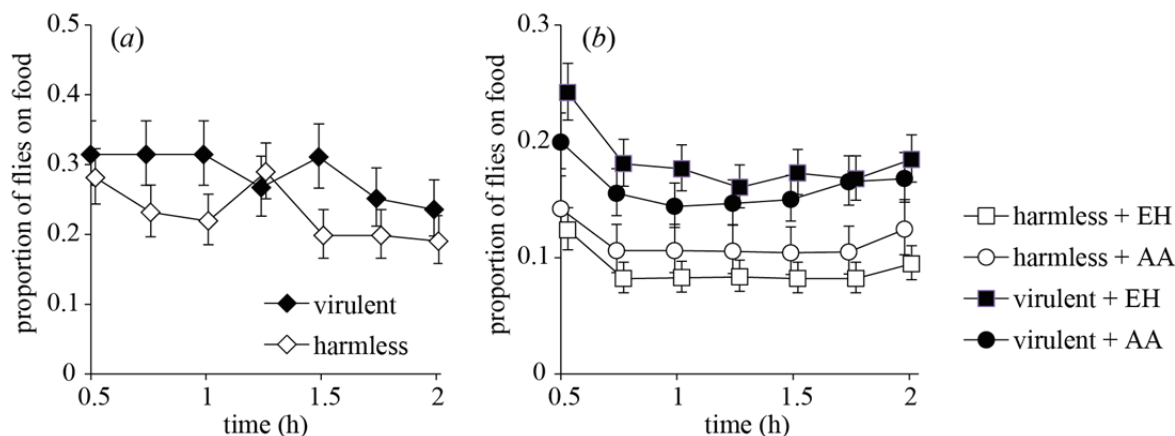


Figure S2. The proportion (mean \pm SE) of flies located on both food substrates among all flies in the cage depending on treatment and time point. (a) Experiment 1; (b) Test phase of experiment 2.

In experiment 1 (figure S2a), the proportion feeding was not affected by the pathogen strain ($\chi^2_1 = 2.42$, $P = 0.12$) but declined with time ($\chi^2_1 = 9.0$, $P = 0.0026$); the slopes of the decline were not heterogeneous between the strain treatments (strain \times time $\chi^2_1 = 0.04$, $P = 0.84$).

In experiment 2 (figure S2b), the proportion feeding change with time in a nonlinear way, with a strong positive quadratic component ($\chi^2_1 = 29.1$, $P < 0.0001$), but the change was parallel across treatment combinations (all interactions with time $P > 0.1$). The proportion feeding was considerably higher for flies conditioned with the virulent strain than for those conditioned with the harmless strain (filled versus open symbols in figure S2b, $\chi^2_1 = 15.4$, $P < 0.0001$). The main effect of the odorant used in conditioning ($\chi^2_1 = 0.2$, $P = 0.67$) and the strain \times odorant interactions ($\chi^2_1 = 2.2$, $P = 0.14$) were not significant. These results suggest that flies infected with virulent *P. entomophila* are more attracted to food, possibly as a result of energetic impact of infection or to counter dehydration.

Excluding confounding effect of proportion of flies feeding on food choice

The above result raises the hypothetical possibility that the differential effect of odour conditioning with the virulent versus harmless pathogen strain on food choice (reported in figure 2 in main text) might be subject to a potential confounding effect of motivation to feed. If this were the case, one would expect to see a relationship between the proportion feeding and the effect of conditioning (i.e., proportion feeding \times odorant interaction) on food choice within strain treatments. We therefore repeated the analyses of food choice in experiment 2 with proportion feeding and proportion feeding \times odorant added to the model as covariates. This analysis upheld all significant results of the original analysis, and the proportion feeding \times odorant interaction was not significant for either treatment (virulent strain: $\chi^2_1 = 0.01$, $P = 0.96$; harmless strain $\chi^2_1 = 2.9$, $P = 0.086$). Thus, the conclusions from experiment 2 reported in the main text are not confounded by differences in the proportion of flies feeding.

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