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Complex behavioural changes after odour exposure in *Drosophila* larvae

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A variety of odorants attract *Drosophila* larvae, although this behaviour can be modulated by experience. For instance, larvae pre-exposed to an attractive odorant may subsequently display less attraction towards the same compound. In previous reports, this phenomenon has been interpreted as a drop in olfactory sensitivity, caused by sensory adaptation. We tried to elucidate the basis of this behavioural modification by pre-exposing larvae to various odours. After multiple pre-exposure cycles larvae were repulsed by initially attractive odours, and pre-exposure did not change the threshold concentration driving a behavioural response. We therefore believe that sensitivity to the odorant was only slightly affected in our protocol. Our results thus do not support the previous interpretation and rather suggest that olfactory pre-exposure induces a change in the hedonic value of the odour. Although we did not succeed in elucidating the exact nature of the underlying mechanism, we can reject an association of the odour with the absence of food as an interpretation of the observed behavioural changes; this is because addition of food did not abolish the repulsion to the pre-exposed odour. In addition to ruling out previous interpretations of odour pre-exposure effects, this study stresses the complexity of *Drosophila* larval behaviour.

Keywords: *Drosphila* larvae; odorants; olfactory learning

Both vertebrates and invertebrates show efficient behaviour in response to biologically relevant olfactory signals. They are able to extract odours related to food, dangerous conditions or mates from a highly complex chemical environment. Accordingly, their sensitivity to background odours is subject to modification, acting mainly through the mechanism of olfactory adaptation. In addition, the actual meaning of an odour is not rigidly programmed, but depends on its context and may change over time. Therefore, the olfactorily driven behaviours of animals tend to adapt to the local environment, notably by olfactory learning. These behavioural modifications, albeit well defined in human psychophysiological assays, are difficult to identify in experiments involving animal models.

Olfactory adaptation is defined by psychophysiologists as a reduction in sensitivity to an odour after repeated or prolonged exposure to that same odour (Dalton 2000). This definition comprises both olfactory adaptation and habituation as defined by Bernhard & van der Kooy (2000), and gives no indication about its cellular basis, that is, sensory adaptation (Zufall & Leinders-Zufall 2000) or central habituation (Wilson 2000). Different properties of olfactory adaptation behaviour have been highlighted. For instance, the degree of adaptation depends on the intensity of the odorant during pre-exposure, and is odorant specific. Indeed, odorant specificity has been used to test discriminative ability in *Drosophila*: a decrease in the response to an odour B after pre-exposure to an odour A has been interpreted as an incomplete discrimination of the two odours (Cobb & Domain 2000; Boyle & Cobb 2005).

Olfactory learning has been studied intensively, in particular in the context of classical conditioning in both vertebrates and invertebrates, using many different approaches (reviewed in Milner et al. 1998; Davis 2005). For instance, in *Drosophila* larvae, olfactory or visual cues (CS; conditioned stimulus) become more attractive after association with a pleasant gustatory stimulus (US; unconditioned stimulus; Scherer et al. 2003; Gerber et al. 2004;

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Honjo & Furukubo-Tokunaga 2005). In some cases, the new behaviour resulting from the association between a CS and a US can be elicited by another stimulus, CS′, similar to the CS. This phenomenon, called generalization, has been used to measure similarity between different and often discriminated stimuli (Ghirlanda & Enquist 2003; Wright & Smith 2004; Guerrieri et al. 2005).

The processes of adaptation and learning (i.e. the loss of sensitivity and a change in hedonic value, respectively) are theoretically clearly distinct, but are empirically difficult to separate. For instance, olfactory adaptation is commonly tested by comparing the olfactory responses of animals pre-exposed to the odorant with the responses of control animals. A lower response is interpreted as a reduced sensitivity to the odour, reflecting olfactory adaptation. However, this lower response could also indicate that the animal values the odour as less positive. Such an effect was demonstrated in a study on *Caenorhabditis elegans* where pre-exposure to an odorant in the absence of food, a protocol previously thought to lead to olfactory adaptation (Colbert & Bargmann 1995), was shown to lead to olfactory associative learning (Nuttley et al. 2002). In this situation, the absence of food acts as a negative US associated with the odorant, leading to a decrease in the chemotactic response towards that odorant.

Drosophila has been used for decades to decode the neural and genetic basis of behaviour. Since the olfactory system of larvae is organized similarly to the adult one despite its limited number of odorant receptor neurons (Kreher et al. 2005; Ramaekers et al. 2005), the fruit fly larva appears to be a promising model system to study olfactory processing. There is evidence for olfactory associative learning in *Drosophila* larvae (Scherer et al. 2003; Hendel et al. 2005; Honjo & Furukubo-Tokunaga 2005). On the other hand, Cobb & Domain (2000) and Boyle & Cobb (2005) used olfactory adaptation of larvae to test olfactory discrimination and, accordingly, proposed models of peripheral olfactory coding. Wuttke & Tompkins (2000) tested larvae mutant for *trp*, a gene encoding a calcium channel whose expression is required during development for olfactory adaptation in the adult (Störtkuhl et al. 1999). They observed no effect of *trp* loss of function in their experimental set-up. However, Wuttke & Tompkins (2000) assumed that only olfactory adaptation was modifying larval behaviour, and did not test for the presence of different forms of learning.

We investigated the mechanisms underlying behavioural changes in *Drosophila* larvae after pre-exposure to odorants, using a modified protocol from Cobb & Domain (2000). We analysed our data in the context of sensory adaptation, increase in sensitivity and associative learning.

GENERAL METHODS

Stocks

Flies from a Canton-S strain (provided by T. Préat, ESPCI, Paris, France) were reared on standard corn food medium at 25°C on a 12:12 h light:dark cycle.

Odorants

Butanol (Fluka cat. 19420, Buchs, Switzerland), hexanol (Fluka cat. 52828), nonanol (Fluka cat. 74278), ethyl caproate (Aldrich/Sigma cat. 14.896-2, Buchs, Switzerland) and ethyl acetate (Merck Schweiz cat. 109623.1000, Dietikon, Switzerland) were used, all highest purity grade. Odorants were displayed on filter paper discs 10 mm in diameter (Schleicher and Schuell cat. 589/2, Bottmingen, Switzerland).

Behavioural Tests

We carried out experiments on agar plates consisting of petri dishes 85 mm in diameter without ergot (Greiner/Huber cat. 632180, Reinach, Switzerland) covered with 2.5% Select Agar (Invitrogen/Lubio Science cat. 30391-023, Lucerne, Switzerland). Sugar and dry yeast plates were covered with 1% Select Agar containing 0.5% autolysed yeast (DIFCO/VWR International cat. 0229-17-6, Dietikon, Switzerland) and 7.5% sugar (from a local grocery store). Yeast plates were produced by covering the surface of the standard agar plates with fresh baking yeast (from a local grocery store) soaked with distilled water. We used young third-instar larvae (75 ± 3 h after egg laying). As no difference was seen between tests done in the morning or afternoon, we pooled all data. Control and experimental groups were always tested in parallel, using larvae from the same culture bottle.

Larvae were washed from the food with 17% sucrose solution. After three rinses in tap water, about 50 larvae were put in a petri dish for 5–15 min. They were then transferred to a pre-exposure plate that contained either an odorant (pre-exposed group) or water (control group) spread on four 10-mm filter paper disks. Filter papers were evenly spaced along the edge of the plate, placed on the agar surface. The amount of odorant indicated below, for each experiment, for the pre-exposure plates relates to the total, i.e. 10 µl corresponds to 4×2.5 µl. Larvae were pre-exposed in the dark for 10 min in a switched-off incubator at room temperature under a fume hood. Then, they were transferred into a clean agar plate for a rest period of 10 min under the fume hood in the presence of light. We carried out this procedure, 10 min of pre-exposure and 10 min of rest, either once or three times.

We carried out the tests as described previously (Heimbeck et al. 1999). Briefly, we placed larvae in the middle of an agar plate containing a pair of filter paper disks on opposite sides, soaked, respectively, with odorant and water. The odorant was put randomly on the left or the right side of the plate. The test plates were then placed under a cardboard cache, in a fume hood. After 5 min, we took a picture of each test plate and counted the larvae. A response index (RI) was calculated: $RI = (N_s - N_c)/(N_s + N_c)$. N_s represents the number of larvae at a distance $d \leq 30$ mm from the odour source. N_c is the number of larvae found inside an identical surface on the opposite side. Positive and negative RIs reflect attraction and avoidance, respectively, and $RI = 0$ indicates indifferent behaviour (tested by measuring attraction towards water). Data presented in the same graph were always from experiments