Olfactory-mediated Behavioral Responses to Lactic Acid Enantiomers in Adult *Drosophila melanogaster*

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Abstract

DL-lactic acid, which is a racemic mixture of D- and L-lactic acid, is a potently attractive odorant in adult *Drosophila melanogaster*. It is unknown, however, whether both enantiomers effectively elicit an olfactory-mediated behavioral response or how potently attractive each enantiomer is compared with other structurally-related chemicals. We quantified the behavioral responses to each lactic acid enantiomer, DL-lactic acid, propionic acid, pyruvic acid, and 2-propanol, using two-choice behavioral assays. L-lactic acid was strongly attractive at several concentrations. D-lactic acid had the same attractiveness at low concentrations, but the attractiveness decreased with increasing concentrations. The difference between enantiomers was supported by the large difference in the dose-response curves for DL- and L-lactic acid at high concentrations. DL- and L-lactic acid elicited strong attractive responses at the identical doses of pyruvic acid and propionic acid, in spite of the low volatility of lactic acid. These findings indicate that lactic acid enantiomers are potently attractive odorants. The different effectiveness between enantiomers suggests that there are enantioselective odorant receptors or odorant binding proteins for lactic acid.

Introduction

For most animals, olfactory systems are crucial for the identification of food, predators, and mates. In olfactory studies, it is difficult to quantify vapor concentrations of individual odorants and compare their effectiveness. Olfactory studies often rely on liquid-phase dilutions to quantify the chemicals tested, even though the associated vapor concentrations constitute the actual stimuli. When presented at an equal liquid concentration as an odor source, individual odorants have different vapor concentrations because of differences in volatility. Therefore, the elicited olfactory responses cannot be compared directly without compensating for, or measuring, the vapor concentrations.

Enantiomeric odorant pairs are particularly fine-tuned probes to investigate olfactory information processing. Some enantiomeric odorant pairs are readily discriminated by humans, monkeys, and rats (Laska and Teubner, 1999; Laska *et al.*, 1999; Rubin and Katz, 2001). Numerous studies indicate that chirality is often essential for the specificity of pheromone perception in insects (Mori, 1996, 1998). Enantiomers have identical non-chiral physico-chemical properties (e.g., saturated vapor pressure, solvent solubility, etc.); they differ only in optical activity and their interaction with other chiral molecules. Hence, any difference in olfactory

responses to enantiomers presented at equal liquid concentrations originates from chiral selectivity at the peripheral level, such as the interaction of each enantiomer with the odorant receptor or odorant binding protein, even if the difference is observed at a behavioral level.

The fruit fly *Drosophila melanogaster*, which is a model animal for the use of powerful genetic techniques, has been used to investigate olfaction at various levels from odorant reception to behavioral response. Individual olfactory neurons within sensory hairs likely express only one or a few of approximately 60 odorant receptor genes (Clyne *et al.*,1999; Vosshall *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 2000; Scott *et al.*, 2001; Dunipace *et al.*, 2001), with the expression of a common receptor-like gene, Or83b (Vosshall *et al.*, 2000). Hallem *et al.* (2004) and Kreher *et al.* (2005) reported an odor response spectrum conferred by fly odorant receptors in vivo based on electrophysiologic single-unit recordings. Using two-photon calcium imaging, Wang *et al.* (2003) reported odor-evoked glomerular activity in the antennal lobe, the first relay station from olfactory neurons to the higher brain centers, such as the mushroom body or lateral horn of the protocerebrum. In addition, olfactory-mediated behavioral responses have been reported in many studies (Devaud, 2003). The odorants used in these studies were often racemic mixtures when the chemicals have chirality. Therefore, it is not known whether the observed response was elicited by a single enantiomer or both. Differences in the olfactory responses to enantiomeric pairs in the fly have not been reported. Screening of the pairs that elicit different responses in the fly is useful for detailed olfactory studies.

The fruit fly is attracted to or repelled by various odors, including acetate esters, organic acids, and alcohols (Barrows, 1907; West, 1961; Fuyama, 1976; Ayyub *et al.*, 1990). Using an olfactometer to measure the behavioral response, Fuyama (1976) reported that DL-lactic acid ($CH_3CH(OH)COOH$), which is a racemic mixture of two enantiomers (D- and L-lactic acid), is a highly potent and attractive odorant in the adult fly, as are ethanol and acetic acid. Lactic acid has very low volatility compared with other volatile chemicals, and is easily degraded during high temperature treatment in gas chromatography, which is used to purify potent volatile odorants or measure vapor concentrations of various odorants in olfactory studies. Although many olfactory-mediated behavioral studies have been reported, no attempts have been made to investigate whether both lactic acid enantiomers are effective or how potently attractive each lactic acid enantiomer is compared with other structurally- related chemicals, such as propionic acid (CH_3CH_2COOH), which is a strong attractive odorant (Ayyub *et al.*, 1990). In this paper, we quantitatively evaluated adult fly olfactory responses to each lactic acid enantiomer using a response index with a two-choice behavioral assay as well as to DL-lactic acid, propionic acid, pyruvic acid ($CH_3COCOOH$), and 2-propanol ($CH_3CH(OH)CH_3$).

Materials and methods

Experimental animals and chemicals

Flies were raised on standard cornmeal-agar-molasses medium at 25 °C. The Canton-S strain was used for all experiments. Adult flies were used for experiments within 1 week after eclosion. All chemicals used as stimulants were analytical grade reagents. For dose- response experiments,

DL-lactic acid (90%), L-lactic acid (90%), propionic acid (98%), pyruvic acid (99.9%), and 2-propanol (98.5%) were purchased from Wako Pure Chemicals Inc. (Osaka, Japan). The other main component in these reagents was water (Hato-oka, Wako Pure Chemicals Inc., personal communication). D-lactic acid (L-0625; 98% in lot we used) and L-lactic acid (L-6402; 99.8% in lot we used) from Sigma Chemical Co. (St. Louis, MO) were used to prepare solutions of equal molar concentrations. All reagent purities were assayed with NaOH titration by the manufacturers. For D-lactic acid, the high purity (98 %) was ascertained by a D-lactate dehydrogenase assay.

Two-choice olfactory behavioral assay

The olfactory behavioral assay was essentially the same as the trap assay used in Higa and Fuyama (1993). Briefly, the assays were conducted in a cage made of a plastic container (300 x 200 x 150 mm) with a tight-fitting lid. The lid had an opening (120 x 60 mm) in the center covered with fine nylon mesh for ventilation. Two glass tubes (15 mm in diameter, 75 mm in height) were placed in opposite corners as a trap; one trap contained 2 ml of odorant solution (diluted in distilled water), and the other one contained the same amount of distilled water as a control. Triton X-100 (0.01%) was added to both traps to drown flies. Before the experiments, flies were transferred into a new culture tube containing standard cornmeal-agar-molasses medium for 24 h, sexed without anesthesia, and maintained in the new tube without any food for 1 h. Approximately 100 flies of either sex were introduced into the cage, which was kept for 24 h without direct illumination of ceiling lights, and most of the flies fell into either one of the traps during this period.

Two-choice gustatory behavioral assay

The gustatory behavioral assay was exactly the same as the two-choice test used in Tanimura *et al.* (1982).

Response index (RI)

The response of the flies was evaluated by an index designated as the "Response index" (RI), calculated as follows. For the olfactory response, RI = (number of flies that entered the odor trap – number of flies that entered the control trap) / number of flies tested. For the gustatory response, RI = (number of flies with blue guts – number of flies with red guts) / number of flies tested. The RI is exactly the same as the "attractability index (AI) described by Fuyama (1976) and the RI described by Ayyub *et al.* (1990). The RI value described in the text is the mean ± SEM. The statistical analysis was performed using a Wilcoxon test. A p value of less than 0.05 was considered to be statistically significant.

Results

Quantification of adult responses to odorants in two-choice olfactory behavioral assay

In the two-choice olfactory behavioral assay, flies that were not starved were free to fly in the cage, land, and walk. From the edge of either trap tube, flies walked downward on the vertical inside

wall of the tube against negative geotaxis, and were trapped in the solution containing Triton X-100, a non-volatile detergent. The behavioral response of the flies was evaluated by an RI index that has been used in many olfactory behavioral studies (Fuyama, 1976; Ayyub *et al.*, 1990; Devaud, 2003). The index theoretically varies between -1 (total repulsion) and +1 (total attraction). An RI of 0 means detection failure or behavioral ignorance of the odorant. All experiments were performed for each sex. In general, the results were similar between males and females. We examined the responses to water, 2 mM sucrose, and 0.35% (v/v) L-lactic acid first (Figure 1A). In the water *vs* water choice assay, flies fell equally into the two water traps. Most of the flies (89%-92%) fell into one of the water traps within 24 h after introduction into the cage, indicating that water (humidity) is an attractant for the adult fly in our assay, and that other factors (light, possible odorants outside the cage, etc.) do not significantly influence the results of the two-choice assay. Thus, RI is also useful to compensate the attractiveness of the solvent. The sucrose *vs* water choice assay revealed that our olfactory assay does not reflect a preference for a gustatory cue. The flies fell equally into the control water and sucrose (2 mM) traps, although sucrose (2mM) strongly elicits the gustatory behavior (Tanimura *et al.*, 1982). In the L-lactic acid *vs* water choice assay, flies mostly fell into the 0.35% L-lactic acid trap (RI; 0.50 ± 0.05, n = 8 for males; 0.54 ± 0.05, n = 8 for females), indicating a strong attractiveness of L-lactic acid. This result is in striking contrast to the result of the gustatory two-choice behavioral assay (Figure 1B). The flies preferred control water intake instead of 0.35% L-lactic acid in the gustatory assay, probably because of the acidity (RI; -0.75 ± 0.16, n = 8 for males; -0.91 ± 0.08, n = 8 for females). These findings indicated that our olfactory behavioral assay detected olfactory-mediated behavior, and

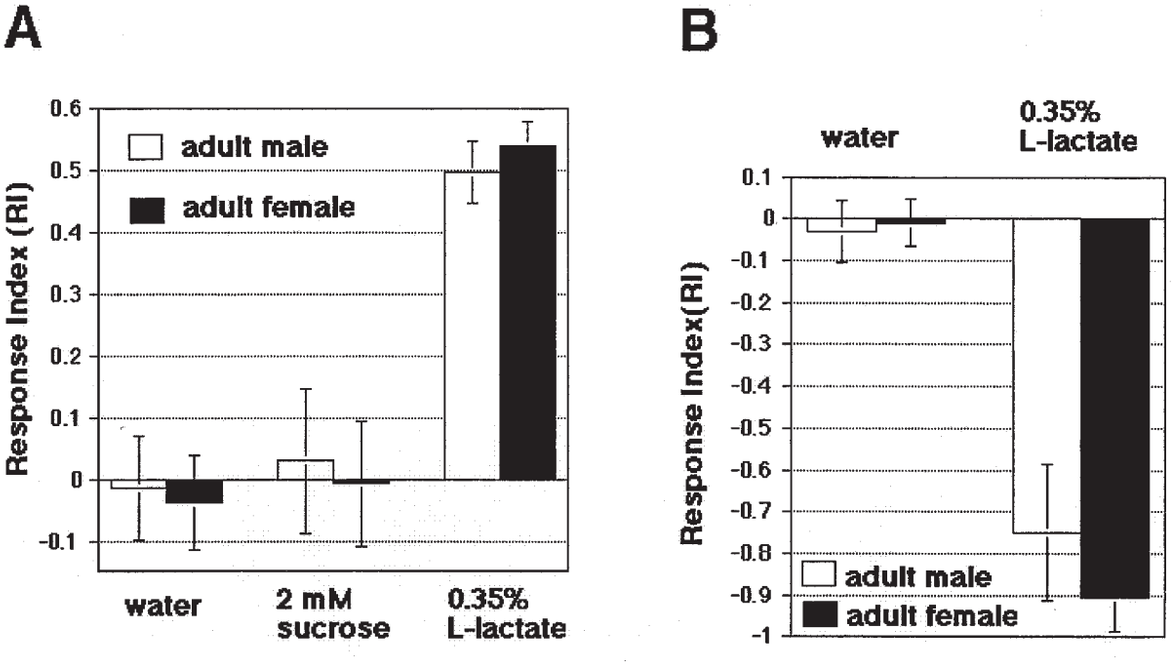

Figure 1 Olfactory-mediated behavioral responses (A) and gustatory-mediated behavioral responses (B) to water, sucrose, and L-lactic acid in adult males and females. Positive and negative RI values denote attractive and avoidance responses, respectively. Vertical bars indicate SEM (n = 8)

not the gustatory-mediated behavior. The attractiveness of L-lactic acid was also observed by using the olfactometer developed by Fuyama (1976) (data not shown).

Comparison of the olfactory responses to DL- and L-lactic acid

DL-Lactic acid is composed of equal amounts of D- and L-lactic acid. If D- and L-lactic acid are equally effective in eliciting an olfactory response, large differences between the responses to DL and L-lactic acid at equal concentrations will not be observed. The dose-response curves for DL- and L-lactic acid are shown in Figure 2. DL- and L-lactic acid were applied in binary dilution steps in concentrations expressed as the base 2 logarithm of percent (v/v) concentration. In general, the results were similar between males and females. Therefore, only the results for females are described below unless otherwise indicated. For DL-lactic acid, the RI increased with increased concentration from an approximate threshold of 0.088% (-3.5 in log scale), showed maximum responses at 0.176% (-2.5 in log scale) to 1.4% (0.5 in log scale), and then decreased toward slight repulsion. With L-lactic acid, the RI increased with increased concentration from an approximate threshold of 0.044% (-4.5 in log scale). The increase of RI was almost the same as that for DL-lactic acid described above, although the RI values to L-lactic acid fluctuated at low concentrations. L-lactic acid was strongly attractive over a wide range of higher concentrations. At high concentrations, there were large significant differences between the responses to DL- and L-lactic acid. For example, the responses to DL- and L-lactic acid at 5.6% (2.5 in log scale) showed slight avoidance (-0.17 ± 0.13, $n = 8$) and strong attractiveness (0.51 ± 0.10, $n = 8$), respectively. Because the RI value to L-lactic acid at 2.8% (1.5 in log scale) was 0.53 ± 0.10 ($n = 8$), the attractiveness derived from the 2.8% L-lactic acid contained in 5.6% DL-lactic acid solution must be inhibited by the coexistent D-lactic acid. These findings indicate that L-lactic acid has strong attractiveness at multiple concentrations, and that D-lactic acid has a different effectiveness in the olfactory behavioral assay as compared with L-lactic acid at high concentrations.

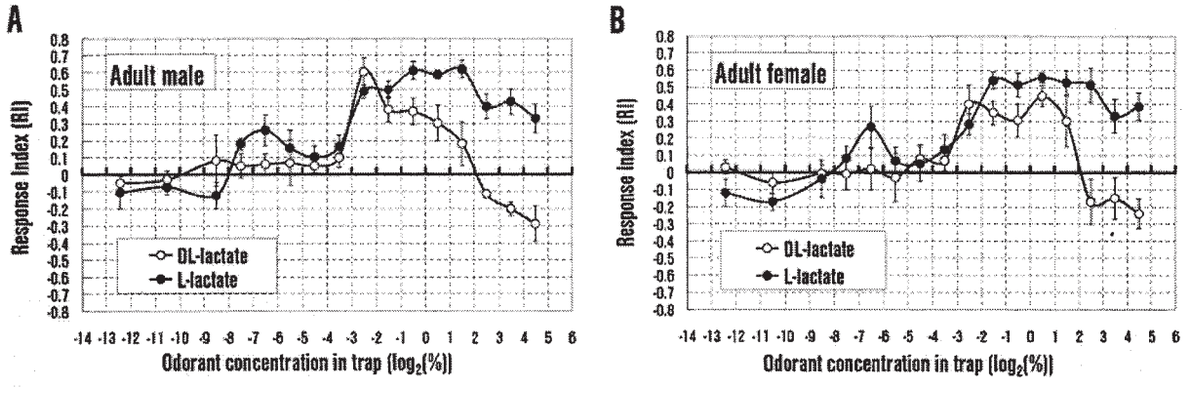

Figure 2 Dose-response curves of adult males (A) and females (B) for DL- and L-lactic acid in olfactory behavioral assay. DL- and L-lactic acid were applied in binary dilution steps in concentrations expressed as a base 2 logarithm of % (v/v) concentration. Vertical bars indicate SEM ($n = 8$).

Comparison of the olfactory-mediated responses to D- and L-lactic acid

The olfactory-mediated responses to D- and L-lactic acid were examined and compared directly. Both lactic acid enantiomers used as stimulants were of the highest purity available. The olfactory-mediated responses to each enantiomer at equal molar concentrations are shown in Figure 3. The responses were examined at 35 mM (corresponds to -1.89 in log scale of Figure 2), 70 mM (-0.916 in log scale), 280 mM (1.09 in log scale), and 560mM (2.09 in log scale). In the D-lactic acid vs water choice assay, D-lactic acid had positive RI values at low concentrations (35 mM and 70 mM). The RI values were comparable to those of L-lactic acid at the same concentrations. The RI value of D-lactic acid, however, decreased at 280 mM, whereas that of L-lactic acid increased. The same tendency was observed at 560 mM. In females, there were significant differences in RI values to D- and L-lactic acid at 280 mM ($p < 0.05$) and 560mM ($p < 0.01$), and there were similar tendencies in males although statistically insignificant. These findings indicate that D-lactic acid has the same attractiveness as L-lactic acid at low concentrations, and that the attractiveness of D-lactic acid decreases with increasing concentrations in females.

Comparison of the olfactory responses to lactic acid and other structurally-related chemicals

The olfactory responses to propionic acid, pyruvic acid, and 2-propanol were examined and compared with the responses to DL- and L-lactic acid at equimolar concentrations. The olfactory responses are shown in Figure 4. Chemicals were applied in binary dilution steps in concentrations expressed as a base 2 logarithm of molar concentration (mol/l) / 0.131 to compare with data presented in Figure 2 because 1% (v/v) lactic acid corresponds to 0.131 mol/l. For propionic acid, the RI gradually increased with increased concentration from an approximate threshold of 1.44 mM (-6.5 in log scale), had maximum responses at 5.76 mM (-4.5 in log scale) to

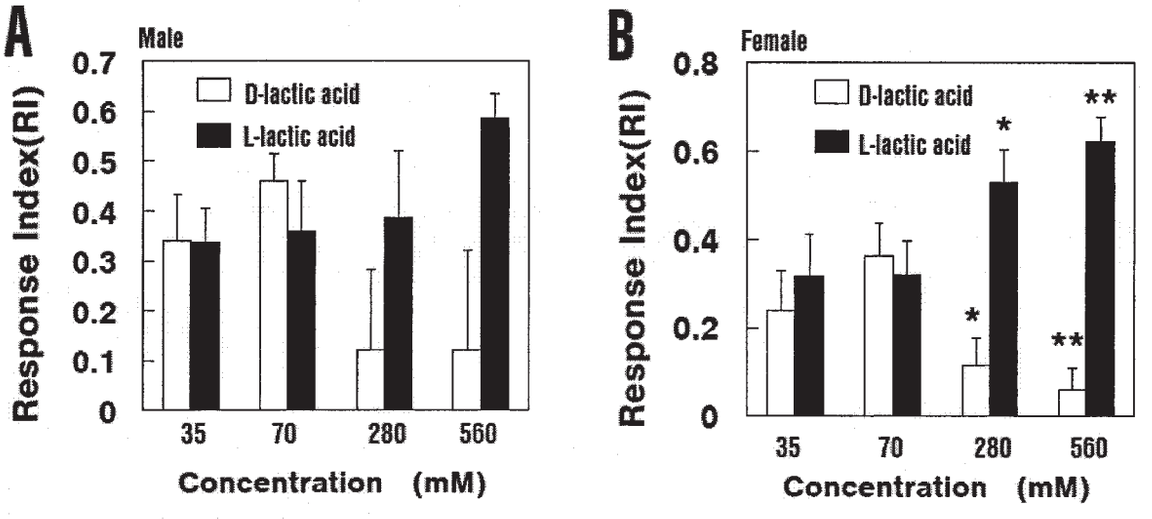

Figure 3 Comparison of olfactory behavioral responses to D- and L-lactic acid for adult males (A) and females (B). Vertical bars indicate SEM (n = 5). Asterisks indicate a significant difference (*; $P < 0.05$, **; $P < 0.01$).

184 mM (0.5 in log scale), and then decreased toward repulsion. For pyruvic acid, the change in RI was almost the same as that for propionic acid, although RI values for pyruvic acid fluctuated at very low concentrations. Pyruvic acid had strong attractiveness over a wide range of higher concentrations. 2-propanol did not induce a significant response. These findings revealed that the maximum values of RI to DL- and L-lactic acid are almost the same as those to pyruvic acid and higher than those to propionic acid (See RI values at 46 mM [-1.5 in log scale] to 184 mM [0.5 in log scale]). The behavioral thresholds for DL- and L-lactic acid are approximately 4- to 8-fold higher liquid-phase concentration (5.6 mM to 11.5 mM ; -4.5 to -3.5 in log scale) compared with those to propionic and pyruvic acids. The actual behavioral thresholds, however, which should be shown as the odorant vapor-phase concentration arriving at the fly, are likely to be comparable between these chemicals because of the low volatility of lactic acid (See discussion). As shown above, DL- and L-lactic acid elicited strong attractive responses at doses identical to those of pyruvic acid and propionic acid. RI values of D-lactic acid were comparable to those of L-lactic acid at low concentrations. It seems clear that each lactic acid enantiomer was highly potent to elicit the olfactory-mediated behavioral response.

Discussion

The use of racemic mixtures of chemicals as odorants often complicates the interpretation of the obtained results because of possible simultaneous stimulation by two odorants. In this study, we examined the olfactory behavioral responses to the enantiomers of lactic acid. The first finding is that each enantiomer elicited the response by itself. The second finding is that there were significant differences between the responses to the enantiomers at equal concentrations in females, and this tendency was also observed in males. These results were supported by the findings of a large difference between responses to DL- and L-lactic acid at high concentrations in male and female. The attractability of L-lactic acid at high concentrations should be reduced by the presence of D-lactic acid for the response to DL-lactic acid. We do not know how many

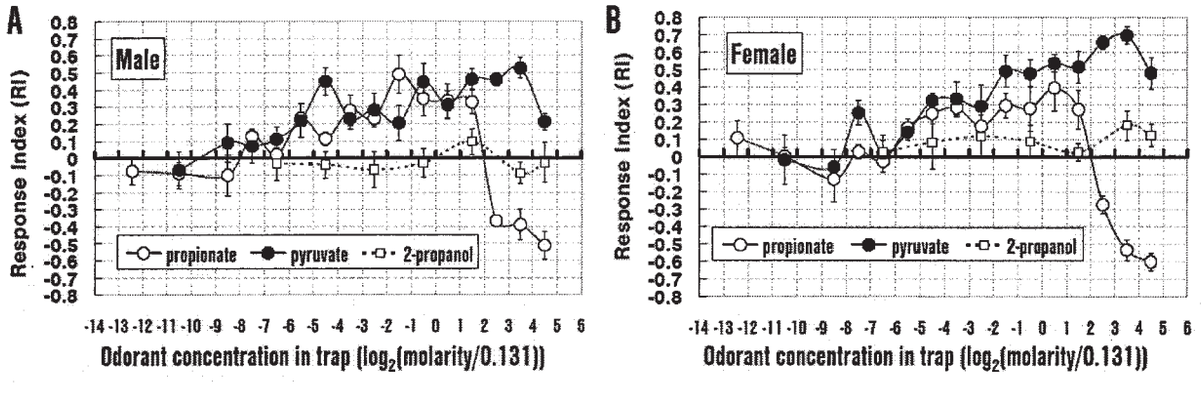

Figure 4 Dose-response curves of adult males (A) and females (B) for the structurally-related chemicals of lactic acid in the olfactory behavioral assay. Chemicals were applied in binary dilution steps in concentrations expressed as a base 2 logarithm of molar concentration (mol/l) / 0.131 to compare this Figure with Figure 2 because 1% (v/v) lactic acid corresponds to 0.131 mol/l. Vertical bars indicate SEM (n = 8).

molecules of odorant reach the olfactory sensory hairs from the odorant solution. A comparable number of odorant molecules, however, should reach the hairs from each enantiomer solution when presented at equal liquid concentrations. Although the molecular, cellular, and neuronal elements that underlie the behavioral response are unknown, the difference between responses to D- and L-lactic acid at equal concentrations suggests the existence of enantioselective odorant receptors or odorant binding proteins for lactic acid.

Next, we compared the response of lactic acid with other structurally-related chemicals. The vapor concentration of an odor, rather than the liquid concentration, directly affects the olfactory response. The behavioral thresholds for DL- and L-lactic acid, shown as the liquid concentrations, were only approximately 4- to 8-fold higher than those for propionic and pyruvic acids, i.e., within one order of magnitude in spite of the low volatility of lactic acid. Saturated vapor pressure is a useful index to judge chemical volatility, although the pressure is for neat chemicals under equilibrium conditions. The saturated vapor pressures of lactic, pyruvic, and propionic acids are 14 mmHg, 200 mmHg, and 410 mmHg at 122 °C, respectively (Windholz, 1983; Chem. Soc. Japan, 1996). At 20 °C, the saturated vapor pressure of pyruvic and propionic acids is 1 mmHg and 2.4 mmHg, respectively (Table 1). Although the saturated vapor pressure of lactic acid at 20 °C is not given in the literature, we estimated the value to be 0.07 mmHg using the ratio of 1: 14: 29-33. Because boiling points of lactic, pyruvic, and propionic acids at 1 mmHg are 85 °C, 21.4 °C, and 4.6 °C, respectively (Windholz, 1983), it is likely that the saturated vapor pressure of lactic acid at 20 °C is much less than 1 mmHg. The loss of weight of neat lactic acid liquid in the trap tube at 25 °C was difficult to measure, whereas that of neat propionic acid liquid was easy to detect several days after the onset of evaporation, indicating the low volatility of lactic acid under non-equilibrium conditions as well as under equilibrium conditions (data not shown). It can be assumed that the number of molecules present in the vapor phase at steady state follows Raoult's law for ideal solutions or Henry's law for ideal dilute solutions. Henry's law works best for solutions with low concentrations of solute and low vapor pressures. One way of expressing Henry's law is that the vapor-phase molar concentration of an odor (solute) above the dilute solution is proportional to the liquid-phase molar concentration at equilibrium conditions. Based on gas chromatography for three dozen chemicals from six homologous chemical series, Cometto-Muniz *et al.* (2003) reported that, as a general rule, there is a simple proportionality between the liquid- and vapor-phase concentrations of these chemicals when they are in equilibrium in a closed container. Their experimental data demonstrated positive correlations between the vapor-phase concentrations and the saturated vapor pressure of structurally-related chemicals at any liquid-phase concentration in general. It is clear that these findings cannot be rigidly applied for diluted odorant solutions under the non-equilibrium conditions we used. It is likely, however, that the number of odorant molecules present in the vapor phase in our experiments was less for lactic acid than for pyruvic and propionic acids when presented at equal liquid concentrations. The actual behavioral thresholds for DL- and L-lactic acid, based on the vapor concentrations, are likely to be comparable to those of pyruvic and propionic acids, or lower. Moreover, the maximum responses to DL- and L-lactic acids were almost the same as those to

Table 1 Values of saturated vapor pressure (P^o) and 5% saturated vapor concentration (SV) in the 20-26 °C range for chemicals we used and acetate esters, which have been used in other olfactory studies of *Drosophila*. Values of P^o at t °C were cited or calculated using the Antoine equation (Windholz, 1983; Chem. Soc. Japan, 1996) except for lactic acid (See discussion). Values of SV at t °C were calculated by the following equation; P^o in mmHg = SV in mol/l x (22.41/273) x (t in °C + 273) x 760 (Dethier and Yost, 1952). Values of 5% SV are shown, at which concentration activation of a small number of glomeruli in the antennal lobe was observed (Wang e*t al.*, 2003).

Chemical	Condensed formula	M.W.	P^o (mmHg)	t (°C)	5% SV (μmol / l)
Lactic acid	$CH_3CH(OH)COOH$	90.1	0.07	20.0	0.192
Pyruvic acid	$CH_3COCOOH$	88.1	1.0	21.4	2.72
Propionic acid	CH_3CH_2COOH	74.1	2.4	20.0	6.57
2-Propanol	$CH_3CH(OH)CH_3$	60.1	32.7	20.0	89.5
Acetic acid	CH_3COOH	60.1	11.6	20.0	31.7
Methyl acetate	CH_3COOCH_3	74.1	172.6	20.0	472.3
Ethyl acetate	$CH_3COO\ C_2H_5$	88.1	75.8	20.0	207.4
Propyl acetate	$CH_3COO(CH_2)_2CH_3$	102.1	24.9	20.0	68.1
Isopropyl acetate	$CH_3COOCH(CH_3)_2$	102.1	60.0	25.1	161.4
Isobutyl acetate	$CH_3COOCH_2CH(CH_3)_2$	116.2	12.8	20.0	35.0
Isoamyl acetate	$CH_3COO(CH_2)_2CH(CH_3)_2$	130.2	5.0	23.7	13.5

pyruvic acid and higher than those to propionic acid. The RI values of D-lactic acid were comparable to those of L-lactic acid at low concentrations. Therefore, it seems clear that each lactic acid enantiomer is also a strong attractant for olfactory -mediated behavior.

Using a Y-maze olfactometer in an olfactory behavioral assay, Ayyub *et al.* (1990) reported that ethyl acetate and propionic acid, as well as isoamylacetate and n-butanol, are strong attractants at low concentrations. The dose-response curves for ethyl acetate and propionic acid by liquid paraffin dilution were almost the same (See Figure 2 in Ayyub *et al.*, 1990). Briefly, both responses began to increase at 10^{-7} dilution, demonstrating high attractiveness near the 10^{-3} dilution (RI = 0.7), and decreased toward repulsion at higher concentrations. The saturated vapor pressure of ethyl acetate is very high compared with that of propionic acid (75.8 mmHg vs. 2.4 mmHg at 20 °C; Table1), indicating the high volatility of ethyl acetate. This finding suggests that the vapor concentration of propionic acid is much lower than that of ethyl acetate when presented at equal concentrations at which comparable responses were elicited. Therefore, the behavioral response might actually be highly sensitive to propionic acid rather than ethyl acetate. These findings suggest that each lactic acid enantiomer is a strong attractant comparable or superior to ethyl acetate in spite of the low volatility of lactic acid.

In olfactory studies, it is difficult to quantitatively compare the effectiveness of responses to individual odorants. The vapor concentrations of individual odorants are affected by the liquid concentration of the odor source and the volatility which is determined by the intrinsic physicochemical molecular properties, as well as to other possible factors, such as the surface area

of the source, affinity to other molecules including solvent, etc. Hallem *et al.* (2004), Kreher *et al.* (2005), and many other investigators have used diluted odorants with the same ratio or a series of liquid dilutions of odorants in paraffin oil or water as stimuli. Wang *et al.* (2003) used an olfactometer, which was a modification of the design described in Dethier and Yost (1952). It permitted quantitative control of odor stimulation by diluting the saturated vapor concentration. However the vapor concentrations of individual odorants were still different in these studies because of different volatility of the liquid phase. For example, the 5% saturated vapor concentrations of several odorants including isoamyl acetate used in Wang *et al.* (2003) are shown in Table 1. Vapor concentration of isoamyl acetate at 5% saturated vapor concentration is 15 times lower and 2 times higher compared with that of ethyl acetate and propionic acid, respectively. When presented as molar concentration, the vapor concentration is directly proportional to vapor pressure. Thus, vapor pressure (mmHg) = vapor concentration in mol/l x (22.41/273) x (temperature in °C + 273) x 760 (Dethier and Yost, 1952). It is necessary to compensate for the difference in vapor concentrations of a series of odorants or to measure the vapor concentrations directly when comparing the stimulating efficiencies. Therefore, relative responsivity to individual odorants of the odorant receptor, glomeruli of the antennal lobe, and behavior should be carefully interpreted. Enantiomeric odorant pairs are useful to overcome these difficulties because they have the same volatility.

We cannot completely exclude the possibility that the high sensitivity and different responses to lactic acid enantiomers were induced by contamination or by- products in the reagents. Each reagent of lactic acid enantiomer is produced from pyruvic acid by the specific lactate dehydrogenase in microbial fermentation, and purified. Although the manufacturers did not provide detailed production methods, we did not detect any pyruvic acid contamination in L-lactic acid reagent by thin layer chromatography in which artificial 1% pyruvic acid contamination in L-lactic acid can be clearly detected (data not shown). High amounts of contamination (12.5 to 24 %; 1/8 to 1/4 of the total) of pyruvic or propionic acid in DL- or L-lactic acid reagents would be needed to explain the threshold difference observed among these chemicals if lactic acid does not show any attractiveness. No chemical is reported to have a higher attractiveness than propionic acid or ethyl acetate in the olfactory-mediated behavioral response (Ayyub *et al.*, 1990). It is unlikely that a potent amount of esters or alcohols is contained in the reagents because of the high purity revealed by the NaOH titration performed by the manufacturers (See Materials and methods).

It is important to question whether the responses to lactic acid enantiomers observed in our analysis are physiologically relevant for odor perception by the organism. Effectiveness of an odor in the natural environment should be carefully evaluated as (1) the concentration in the odor source, such as food, (2) the volatility of the odor, and (3) fly's sensitivity to the odor. The olfactory-mediated behavioral response we examined showed high sensitivity to lactic acid enantiomers in spite of low volatility. How do the amounts of lactic acid contained in natural foods of Drosophila, such as fermented fruit or vegetables, compare with those of other odorants? Using gas chromatography, Umano *et al.* (1992) reported that esters constituted over 80% of the total

volatiles in ripened pineapple, and that ethyl acetate constituted 33% of the volatiles. The yield of total volatiles, however, was only 0.0009% (w/w), i.e., 9 ppm. Using head space gas chromatography, Ashida *et al.* (1987) reported that the concentration of isoamyl acetate in wine (fermented grape by wild-type yeast) is only 10.9 ppm, whereas alcohol constitutes 12.8%. Using high-performance liquid chromatography with electrochemical detection, Kotani *et al.* (2004) reported that concentrations of lactic acid in commercial wines are 0.30 to 2.24 g/L (3.3 to 24.9 mM), indicating that there is a considerable amount of lactic acid without lactic acid fermentation. High performance liquid chromatography analysis demonstrated that vegetable juice medium fermented by lactic acid bacteria contains 0.4 % to 1% (w/v) lactic acid (Gardner *et al.*, 2001). Behavioral responses at these concentrations were observed in our assay. Our behavioral assay, as in all other behavioral assays, is limited by the sensitivity. In their natural environment, flies encounter a vast array of odorants originating from rotting fruits or vegetables, fly to, and land near the food. These behaviors should be triggered by a combination of individual odorant perceptions. Therefore, the behavioral response to a single odorant in our assay might be strongly triggered at very high concentrations, which are never experienced in the native environment although olfactory receptor activation might be triggered at lower concentrations. In fact, the odorant mixtures are more effective than any one compound (West *et al.*, 1961).

A number of extensive studies on the relationship between Drosophila and naturally occurring yeast species were performed during the 1950s by Dobzhansky and colleagues (da Cunha *et al.*, 1951, 1957; Dobzhansky and da Cunha, 1955; Dobzhansky *et al.*, 1956; Cooper, 1960). The behavioral responses to ethanol, acetic acid, and propionic acid, which are the final products in microbial fermentation, were repeatedly reported. In spite of the earlier olfactory studies indicating lactic acid as an attractant (Barrows, 1907; Fuyama, 1976), little attention has been paid to the relationship between the olfactory behavior of Drosophila and lactic acid, the final metabolite of lactate fermentation. It is interesting that female mosquitoes, which is the same order (Diptera) as Drosophila, are repeatedly reported to be attracted to L-lactic acid, present on human skin as well as in human breath. L-lactic acid has an essential role in the attractiveness of human skin odor because without this compound the remaining volatiles from the skin are not effective (Geier *et al.*, 1996). The synergistic abilities of L-lactic acid and other odorants to act as attractants have been demonstrated in several behavioral studies (Acree *et al.*, 1968; Smith *et al.*, 1970; Eiras and Jepson, 1994; Steib *et al.*, 2001). Electroantennogram responses have been obtained for L-lactic acid as well as for other human sweat components (Costantini *et al.*, 2001). In Drosophila, the biologic meaning of high sensitivity of the behavioral response to lactic acid and different effectiveness of lactic acid enantiomers requires further elucidation.

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