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Phosphatidylcholine profile-mediated group recognition in catfish

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Summary

Animal groups are integrated by emission of discrete signals from members, so-called social signals, which have evolved for each species. Among communication signals, chemical signals play an important role for recognition of group membership. The catfish *Plotosus lineatus* forms a dense school immediately after hatching, and school recognition is under the control of chemical signals emitted by the school members. The key substance(s) governing this recognition are deduced to be a mixture of phosphatidylcholines (PC). To substantiate this hypothesis that a mixture of PC molecular species functions as recognition of school-specific odor, we examined the ability of *P. lineatus* to discriminate between familiar and unfamiliar PCs. *P. lineatus* responded only to PCs from a familiar school, and not to those from unfamiliar schools. PC molecular species were then analyzed by quantitative high performance liquid chromatography, which resulted in not only a complex mixture of PC molecular species, but also school-specific PC profiles. Furthermore, multivariate analysis of the quantified PC peaks revealed the presence of various PC profiles. Finally, we showed that the modification of PC profiles disrupts the recognition of school odor in *P. lineatus*. Therefore, we conclude that the recognition of school odor in *P. lineatus* is governed by school-specific PC profiles.

Supplementary material available online at
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Key words: chemical signal, recognition system, social organization.

Introduction

The social organization of animals relies on careful recognition of group membership. In particular, the ability to recognize familiar individuals (such as mate, kin and infant) is necessary to integrate complex social organization (Bradbury and Vehrencamp, 1998). Recognition between members is often dependent on distinct chemical signals that are emitted from them (Wyatt, 2003). Although the chemical signals governing sexual behaviors have been well characterized in insects and in mammals, little is known about the chemical cues that provide information about cognitive processes such as identifying the characteristics of individuals (Dulac and Torello, 2003). Such signals must be the key to investigating the mechanisms that govern not only perception and cognition, but also social behaviors elicited by natural stimuli (Dulac and Torello, 2003; Schaal et al., 2003). Therefore, identification of the salient cues is important for understanding a wide variety of animal communications.

Recent advances in olfactory research have provided the opportunity to identify unknown chemical signals involved in communications (Brennan and Zufall, 2006). It is well known that the individual status of conspecifics is discerned by a complex mixture of chemicals, as reported in a wide range of animals from invertebrates to vertebrates (Dulac and Torello, 2003). In social insects, cuticular hydrocarbons can convey information of individual status, by which their stable society is organized (Steinmetz et al., 2003; D'Ettorre and Heinze, 2005). Likewise, in higher vertebrates, complex mixtures of metabolites are used for the identification of other individuals (Dulac and Torello, 2003; Brennan and Zufall, 2006). In most cases, however, signal identity among chemical mixtures is poorly understood.

The catfish *Plotosus lineatus* is a good model for studying group (school) recognition *via* chemical signals (Krause and Ruxton, 2002). Soon after hatching, *P. lineatus* forms a school known as a clutch (Kinoshita, 1975; Matsumura, 2004; Moriuchi and Dotsu, 1973; Golani, 2002) (see Fig. S1 in supplementary material), which appears as a highly cohesive ball-shape and has a group organization based on familiarity. When two schools encounter each other, they either merge into one school, or re-

form the separated schools, depending on the situation (Kinoshita, 1975; Matsumura, 2004). Similar dynamic group organization has been reported in many animals whose group members recognize each other through various sensory systems, such as vision, audition, somatosensation and olfaction (Kerth and König, 1999; Barber and Ruxton, 2000; Krause and Ruxton, 2002; Parrish et al., 2002; Camazine et al., 2003). Schooling in *P. lineatus* is also organized by the visual, lateral-line and chemical senses (Sato, 1938; Kinoshita, 1975; Hayashi et al., 1994). Discrimination between familiar and unfamiliar schools in *P. lineatus* is governed by a chemical signal originating from school members, enabling them to discriminate between odors of familiar and unfamiliar schools (Kinoshita, 1975; Hayashi et al., 1994). In addition, chemical signals for school recognition are perceived through olfaction because naris-occluded individuals were not attracted to seawater in which the familiar school had been maintained (Hayashi et al., 1994). However, the chemical signal underlying school recognition is yet to be elucidated. Previously, we established a behavioral bioassay to estimate the recognition of school odor in individual fish (Matsumura et al., 2004). Using the bioassay, we deduced that school odor is a mixture of phosphatidylcholine (PC) molecular species; however, its precise functions remained unsolved (Matsumura et al., 2004). Therefore, the present study asks: (1) are PCs alone sufficient to elicit and be solely responsible for recognition of school odor? (2) Does *P. lineatus* discriminate between familiar and unfamiliar PCs? (3) Are PC profiles indispensable to expression of recognition activity of school odor?

Materials and methods

Experimental animals

Schools comprising several hundreds of juvenile catfish *Plotosus lineatus* Thub (approximately 3 cm in total length) were collected in Aburatsubo Inlet, Kanagawa, Japan from July to September, during 2000–2005. Each school was separately maintained in a 1000-l polyethylene tank with running natural seawater and fed *ad libitum* with krill *Euphausia superba* and the Japanese sardine *Engraulis japonica* at the Misaki Marine Biological Station of the University of Tokyo; the schools were thus maintained in a healthy state for 1 month.

Extraction and isolation of PC

Preparation of PC from *P. lineatus* was essentially as described previously (Matsumura et al., 2004). Ethanol extract of skin mucus collected from school members was partitioned between water and chloroform, and the chloroform layer was loaded onto a polystyrene column (TSK G3000S, Tosoh, Tokyo, Japan; 70 mm×10 mm i.d.) and eluted with 50 ml each of 70%, 90%, 100% methanol, followed by chloroform/methanol (1:1). The fractions eluted with the last three solvents were combined and separated by HPLC on a polyamide column (250 mm×20 mm, YMC Pack Polyamide II, Yamamura Chemical Laboratories, Kyoto, Japan) at a flow rate of 3.5 ml min^{-1} with acetonitrile/methanol/water (73:25:3 v/v) to yield a PC fraction. This material was further separated by HPLC on an ODS column (Phenomenex Luna 5 μ, 250×10 mm, Phenomenex, Torrance, CA, USA) at 3 ml min^{-1} with 100% methanol into Fraction 1 and Fraction 2.

Bioassay

Procedures for bioassay and data analysis were essentially the same as described previously (Matsumura et al., 2004). In brief, a test fish, chosen at random from a test school, was introduced into a test device (68 cm×40 cm×15 cm; seawater, 3 cm depth) and acclimated for 3 min (i.e. to constant swimming behavior). Agar blocks (1 cm^3) containing a test solution and the solvent (methanol) were attached to each side of the container using a metal wire (2 cm long×0.1 cm diameter). *P. lineatus* presented a characteristic behavior towards the agar blocks that contained the school odor (i.e. the fish returned to the agar blocks after first passing them). The response of the test fish to the agar blocks was video recorded for 5 min. The positions of the treatment and control agar blocks were switched during successive trials. Test individuals, test seawater and stimulants were replaced at each trial. The number of trials is given in Table S1 in supplementary material. All samples were assayed blind. All behavioral assays were carried out at a PC concentration of 0.15 mg ml^{-1}, except for the dose–response experiment shown in Fig. 1. To assess the ability of *P. lineatus* to distinguish between PCs obtained from two schools (school A and school B), the following combinations of samples were used: school A *vs* control (methanol), school B *vs* control, school A *vs* school B, and control *vs* control. In order to modify the PC profile, PC was supplemented with 0.1, 1 and 10% (w/w) of either 1-palmitoyl-2-(4,7,10,13,16,19-(*E*)-docosahexaenoyl)-sn-glycero-3-phosphocholine [16:0-22:6 (SPC1)] or 1-hexadecanoyl-2-(5,8,11,14-(*E*)-eicosatetraenoyl)-sn-glycero-3 phosphocholine[16:0-20:4 (SPC2)] (Sigma, St Louis, MO, USA).

Data analysis

The data obtained from these assays were evaluated as described previously (Matsumura et al., 2004). In brief, the number of passes to the agar blocks (*N*) and turns to the agar blocks (*n*) were counted; a turn was counted only when the test fish returned to the agar block within 5 s of passing it. The percentage of turns (RT) was calculated using the following equation: RT=(*n*/*N*)×100. RT was transformed to arcsine values using the following equation: transformed value (TV, degree)=arcsine (RT/100)1/2 (see supplementary material Table S1 for the statistical analysis between TVs) (Zar, 1999). To compare the activity between each stimulus, a response index (RI) was calculated using the following equation: RI=[(RTT–RTC)/(RTT+RTC)]×100, where RTT is the RT obtained for the treatment agar block and RTC is that for the control agar block. RI of each sample was compared using a two-sample *t*-test. The statistical analysis was performed using the computing software R (http://www.r-project.org). In all figures, values are means ± standard error of the mean (s.e.m.).

Analysis of PC molecular species

A diethyl ether/ethanol (95:5, v/v) solution of PC (1 mg in 500 μl) was added to 100 μl of buffer (100 mmol l^{-1} Tris-HCl, 20 mmol l^{-1} $CaCl_2$, pH 7.3) containing 0.5 mg of *Clostridium perfringens* phospholipase C Type XIV (PLC; Sigma), and the mixture was incubated at 37°C for 2 h. The solution was extracted with diethyl ether (3×300 μl), and the ether extracts were dried *in vacuo* to obtain 1,2-diacyl glycerol (1,2-DG). 1,2-DG was dissolved in 500 μl dry toluene (Wako Pure Chem, Osaka, Japan) and 30 μl dry pyridine (Wako Pure Chem), and 3,5-dinitrophenyl isocyanate (3 mg; Aldrich, St Louis, MO, USA) was added to this solution. The mixture was allowed to stand for 1 h at room temperature. To quench the reaction, methanol (100 μl) was added to the reaction mixture; the solvents were removed in a stream of nitrogen gas. The residue was purified using TLC (Silica Gel 60 F254, 8×20 cm, 0.25 mm thick; Merck, Whitehouse Station, NJ, USA). The TLC plate was developed with a mixture of *n*-hexane/dichloromethane/ethanol (40:10:3, v/v/v). A band corresponding to the 1,2-DG derivatives (R_f, 0.58) was scraped off and extracted with diethyl ether. The ether extract was dried *in vacuo* to furnish the dinitrophenylurethane (DNPU) derivatives of 1,2-DG (Okabe et al., 1999).

The PC DNPU derivatives [10 μl (1 mg ml^{-1} methanol)] were analyzed by an HPLC system consisting of a Waters 2695 Separations Module and Waters 2996 photodiode array (PDA) detector (Waters Corporation, Milford, MA, USA). The following five columns were connected for the isolation of PC components: Prodigy ODS-3 (Phenomenex), Luna 5 μ C18(2) (Phenomenex), TSKgel ODS-80Ts (Tosoh), two Inertsil ODS-3 (250×4.6 mm each; GL Sciences, Tokyo, Japan). The column temperature was maintained at 23°C; the column was eluted with acetonitrile/isopropanol (8:2) at a flow rate of 0.5 ml min^{-1}, and peaks were detected by UV absorption at 254 nm. The recordings of chromatogram and the quantitative analysis of peak areas were analyzed using Waters Empower PDA software (Waters Corporation, Milford, MA, USA; see Fig. 2). Fatty acids in PC were identified by a combination of mass spectrometric (JMS-700T; JEOL, Tokyo, Japan) and gas chromatographic (GC) analysis. In brief, fast atom bombardment (FAB) mass spectra of the isolated PC DNPU derivatives were recorded using *m*-nitrobenzyl alcohol as a matrix (Fig. 4A). The PC DNPU derivative, isolated by HPLC, was dissolved in 1 ml of 0.5 mol l^{-1} sodium methoxide. The solution was kept at 50°C for 10 min. After cooling to room temperature, the reaction mixture was partitioned by adding 0.1 ml of acetic acid and 4 ml each of water and *n*-hexane. Subsequently, the aqueous layer was extracted twice with *n*-hexane. The extracts were dried in a stream of nitrogen gas, dissolved in *n*-hexane, and analyzed by GC using a Shimadzu GC-9A gas chromatograph on SPTM-2380 column (30 m×0.25 mm i.d.; Supelco, St Louis, MO, USA). Helium was used as a carrier gas and temperature was maintained at 140°C for 5 min followed by programmed increases of 3°C min^{-1} up to 240°C (Fig. 4B). The structures were determined by comparing the retention time of standard fatty acid methyl esters.

Principal component analysis (PCA) and visualization of correlation matrix with PC profile

The relative peak areas of the 19 selected peaks obtained in the HPLC chromatogram of dinitrophenylurethane (DNPU) derivatives of PC molecular species were re-standardized to 100% and transformed following Aitchison's formula (Aitchison, 1986), $Z_{ij}=\ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area i for school j, Y_{ij} is the peak area i (No. 1–19) for school j (47 schools), and $g(Y_j)$ is the geometric mean of peaks for school j. The transformed areas were used as variables in a PCA operated by a custom program with R (http://www.r-project.org) to obtain the principal component scores and proportions. A two-dimensional (2D) space was spanned by the two leading principal components (PC1 and PC2; Fig. 5E). The schools (47 schools in total) were again sorted on the basis of the first principal component (PC1) to represent a square correlation matrix (Fig. 5F). The figure is symmetrical about the diagonal of those in which the darker black colors represent lower correlations and the lighter colors, higher correlations.

Results

Dose-dependent response to skin mucus and PC

To substantiate our previous report (Matsumura et al., 2004), which suggested that school odor is a mixture of PC molecular species, we first compared the turn behavior-eliciting activity of the skin mucus and the PC isolated from the skin mucus. The dose–response curves for skin mucus and PC, ranging from 0.0015 to 0.3 mg ml^{-1}, were not significantly different [two-way ANOVA: $P<0.05$ (N=7–12; d.f.=4, $F_{4,94}=3.305$, $P=0.0146$ for doses; d.f.=1, $F_{1,8}=0.0144$, $P=0.905$ for skin mucus *vs* PC in Fig. 1; for the number of turns, see Table S1 in supplementary material)]. Thus, PC elicits the typical turn behavior in *P. lineatus* as efficiently as the skin mucus. In contrast, egg yolk- and soybean-derived PCs did not elicit turn behavior in *P. lineatus* (K.M., S.M. and N.F., unpublished

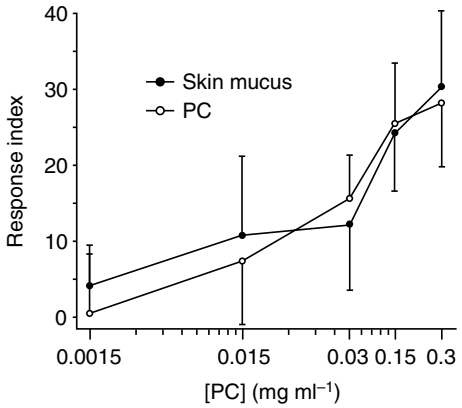

Fig. 1. Dose-dependent response of *P. lineatus* to skin mucus (closed circles) and PC (open circles) obtained from familiar school using the two-choice test based on turn behavior. Statistical comparison between dose responses revealed that skin mucus and PC have non-significantly different activity. Values are means ± s.e.m.

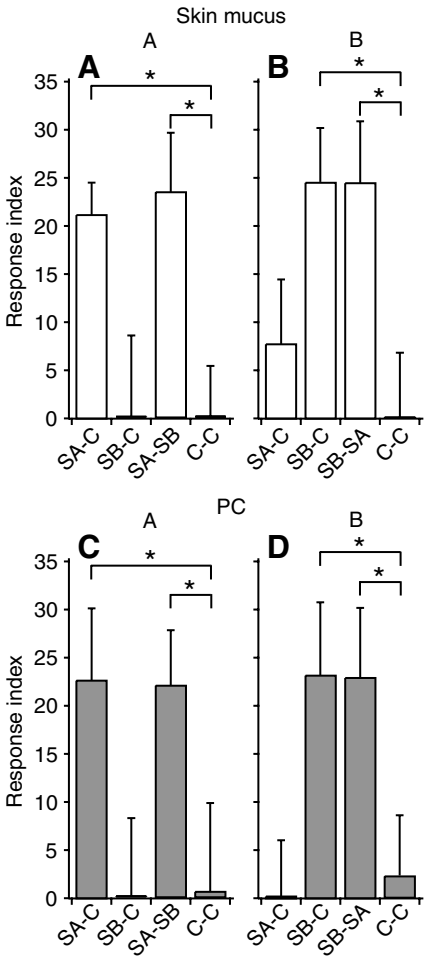

Fig. 2. School-specific responses of *P. lineatus* to skin mucus (A,B) and PC (C,D) obtained from schools A (SA) and B (SB). (A) Response of SA to skin mucus; (B) Response of SB to skin mucus; (C) Response of SA to PC; (D) Response of SB to PC. Sample combinations tested are school A *vs* solvent control (SA-C), school B *vs* solvent control (SB-C), school A *vs* school B (SA-SB), and solvent control *vs* solvent control (C-C). Asterisks indicate a significant difference at $P<0.05$ (two-sample *t*-test).

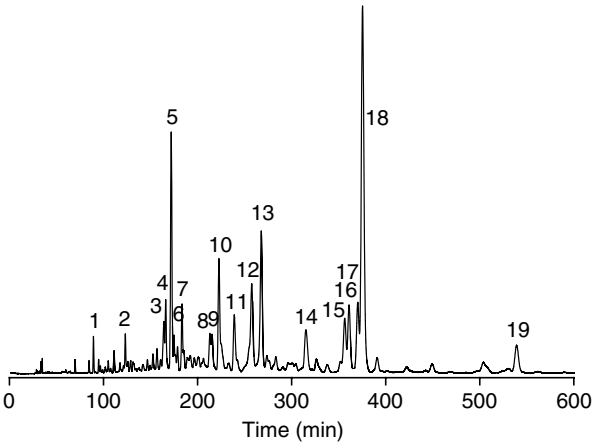

Fig. 3. Quantitative HPLC analysis of the PC molecular species revealed that skin mucus of *P. lineatus* includes a large number of PC molecular species. Selected peaks 1–19 were identified and the relative peak areas used for multivariate analysis.

results), suggesting that PC originating from the skin mucus is essential for recognizing school odor.

Discrimination between familiar PC and unfamiliar PC

P. lineatus was reported to discriminate between the odors of familiar and unfamiliar schools (Kinoshita, 1975). Therefore, we examined whether *P. lineatus* can recognize familiar PC using the two-choice test (School A *vs* School B). The results showed that a school member clearly selected the skin mucus of the familiar school rather than the control or the skin mucus of the unfamiliar schools (N=8–13; two sample *t*-test, $P<0.05$; Fig. 2A,B; for the number of turns, see Table S1 in supplementary material), indicating that *P. lineatus* distinguishes between familiar and unfamiliar mucus. This is also true for PCs; the school members selected the PC of the familiar mucus but not that of the control or the unfamiliar PCs (N=9–15; two sample *t*-test, $P<0.05$; Fig. 2C,D; for the number of turns, see Table S1 in supplementary material). These results indicate that *P. lineatus* discriminates between the odors of familiar and unfamiliar schools by their PC content.

Identification of PC molecular species

From the results described in the previous section, we hypothesized that a difference of PC molecular species between schools is critical to recognize the familiar school. To compare PC profiles between schools, we first attempted an analysis of the PC molecular species. The PC derivatives were separated by high performance liquid chromatography (HPLC) equipped with a photodiode array detection system, which showed approximately 60 peaks, including 10 prominent peaks (Fig. 3). The structures of the major peaks, 1–19, were determined by a combination of mass spectrometry and gas chromatography (Table 1). In the case of peak 18 (Fig. 4A,B), ion peaks at *m/z* 522 and 548 in the mass spectrum could be assigned to acyl groups linked to sn-1 and -2 positions of glycerol, respectively (position is interchangeable), and a peak at *m/z* 577 originated from a dinitrophenylurethane group (Fig. 4A), while GC analysis identified such fatty acids as 16:0 and 18:1(n-9), respectively (Fig. 4B), thus corroborating that the structure is 16:0-18:1(n-9). Some of the peaks could not be completely determined due to an inseparable mixture of PC species and/or scarcity for analysis (Table 1).

Heterogeneity of PC profile

Using quantitative HPLC analysis of PC molecular species, as shown in Fig. 4, we examined HPLC profiles of PCs prepared from 47 schools. The results clearly showed that schools contained a similar set of PC molecular species, but their relative proportions varied from school to school (see selected example shown in Fig. 5A–D), indicating that each school has a specific PC profile. To confirm the diversity of

Table 1. *Chemical structure of phosphatidylcholine in peaks 1 to 19*

Peak no.	Side chain	Peak area (%)
1	20:5(n-3), 22:6(n-3)	0.86±0.27
2	16:1(n-7), 20:5(n-3)	1.13±0.12
	18:3(n-4), 20:5(n-3)	
3	18:1(n-7), 20:5(n-3)	2.35±0.28
4	18:1(n-9), 20:5(n-3)	3.27±0.54
5	16:0, 20:5(n-3)	8.05±0.73
6	18:1(n-9), 22:6(n-3)	1.81±0.26
7	16:0, 22:6(n-3)	2.45±0.42
8	ND	1.96±0.24
9	18:1(n-9), 20:4(n-6)	2.21±0.38
10	16:0, 20:4(n-6)	5.80±0.93
11	16:3(n-4), 20:5(n-3)	2.94±0.41
12	ND	6.51±0.70
13	16:0, 16:1(n-9)	7.69±1.15
	18:1(n-9), 18:3(n-4)	
14	ND	3.90±0.66
15	18:1(n-7), 18:1(n-9)	1.96±0.49
16	18:1(n-9), 18:1(n-9)	6.47±1.09
17	16:0, 18:1(n-7)	4.79±0.63
18	16:0, 18:1(n-9)	30.57±1.35
19	16:3(n-4), 18:1(n-9)	2.79±0.48

Peak areas were used for principal components analysis. Values are means ± s.d. ND, not determined.

PC profiles between schools, we conducted principal component analysis (PCA) of 47 schools.

The multidimensional PCA data were projected onto a two-dimensional space spanned by the two leading principal components (Fig. 5E); selected profiles of Fig. 5A–D (boxed) are plotted in scattered positions onto a two-dimensional space. PCA revealed distinct variations in the PC profiles without a specific distribution pattern among the schools. In other words, each school had a school-specific PC profile. Moreover, the overall visual representation of the similarities between PC profiles among schools was carried out after sorting the schools based on PC1, in which the school-by-school correlation matrix was graded by tones varying from black to white (Fig. 5F). The correlation matrix clearly showed PC1-based graded similarities among the PC profiles of the schools whose correlation coefficients range from 0.656 to 1 (1 meaning auto correlation). For example, the correlation coefficient between the profiles of Fig. 5A,D is 0.875 (A *vs* D=0.875). Thus, the PC profile, comprising remarkably diverse molecular species, is the signature of each school, suggesting its involvement in school recognition.

Responsibility of PC profile

To address whether *P. lineatus* recognizes a single PC molecular species or PC profiles, we divided the PC molecular species into two fractions using HPLC (Fig. 6A) and evaluated the turn behavior caused by each fraction (Fig. 6B). Neither fraction (Fr.) 1 nor Fr. 2 elicited turn behavior, while the re-constructed PC (mix) was as active as the original PC

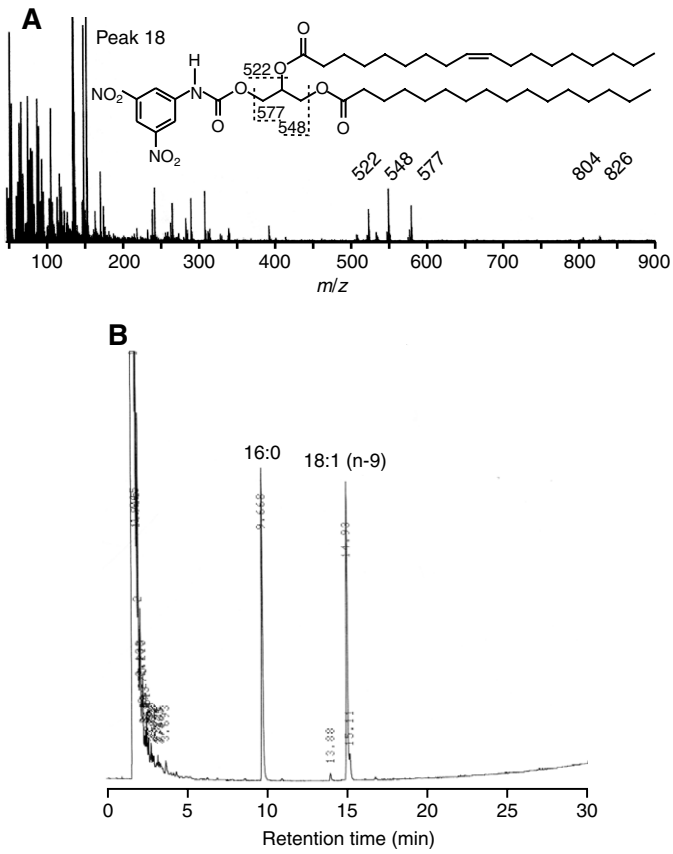

Fig. 4. Identification of PC molecular species. The PC molecular species of peak 18 is identified by a combination of (A) mass spectroscopy and (B) gas chromatography. *y*-axis, signal intensity.

(approximately 90% identical, N=9–11; P<0.05; Fig. 6B; for the number of turns, see Table S1 in supplementary material). This result indicated that at least two PC molecular species are involved in school recognition. In other words, the result suggests that school-specific recognition is responsible for the profile of more than two PC molecular species. We then modified the PC profile by adding two synthetic PCs, SPC1 [16:0-22:6(n-3)] and SPC2 [16:0-20:4(n-6)], which are known to be present in skin mucus of *P. lineatus*. Addition of 10% (w/w) of either SPC1 or SPC2 significantly diminished the activity of the original PC (P<0.05), while approximately 50% of the original activity was lost by adding 1% SPC1 or SPC2 (Fig. 6C; for the number of turns, see Table S1 in supplementary material). The addition of 0.1% SPCs did not affect the activity (P<0.05; Fig. 6C). Incidentally, neither SPC1 nor SPC2 individually showed the activity (Fig. 6C). These results indicate that the profiles of PC molecular species are responsible for the activity. Thus, we concluded that PC profile encodes school recognition in *P. lineatus*.

Discussion

Chemical profile-based recognition signal

Our previous finding (Matsumura et al., 2004) suggested that

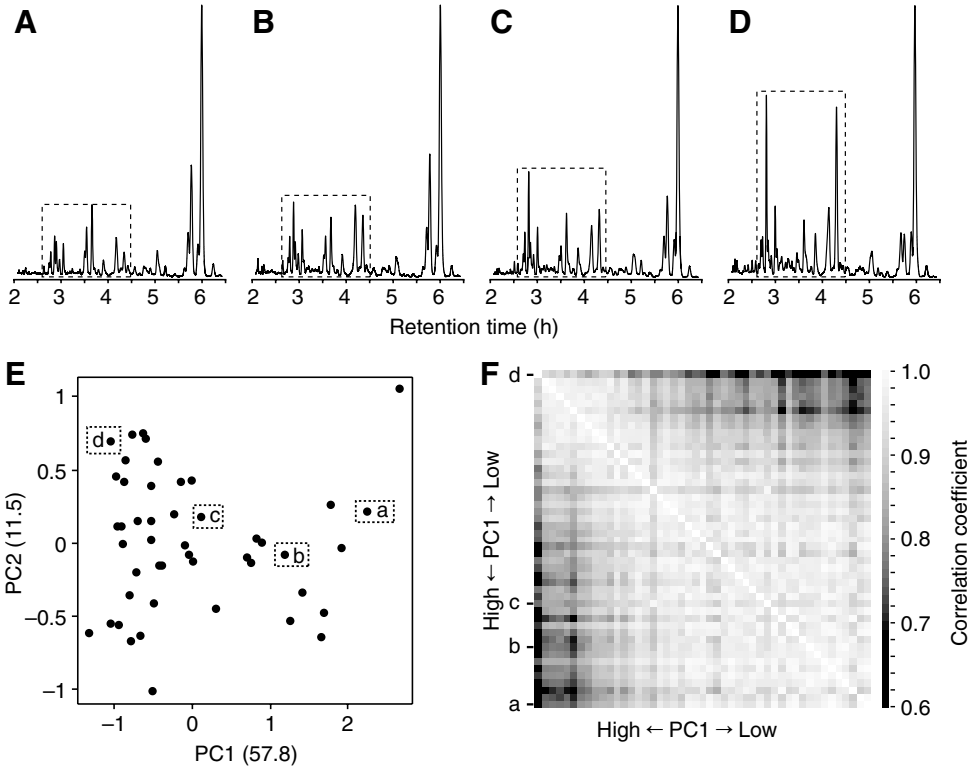

Fig. 5. Inter-school variations in the PC profile. (A–D) HPLC chromatogram of PC obtained from four schools showed a heterogeneous PC profile, particularly the peaks in the boxed frames. (E) The two leading principal components (PC1 and PC2) were projected into 2D space. Each dot indicates the schools that were plotted without any characteristic distribution pattern. (F) Comparison of PC profiles by representing a matrix of correlation coefficients that indicate the degree of overall similarity. Black colors indicate lower correlations.

the composition of PC molecular species is different from school to school, allowing *P. lineatus* to discriminate their own school from others. To support this hypothesis, we attempted in this study to demonstrate that recognition of school odor in *P. lineatus* is determined by a school-specific PC profile constructed from its diverse molecular species. First, we showed that *P. lineatus* can clearly discriminate between the odors of a familiar school and unfamiliar ones using PC, indicating that PC is a reliable signal for the recognition of a familiar school. To reveal school-specific PC profiles, PC molecular species were analyzed by a combination of HPLC and mass spectrometry. Each school contained the same set of PC molecular species, but not the same specific PC molecular species (Fig. 5A–D). In addition, the mixing pattern of the PC molecular species (PC profile) was highly heterogeneous between schools, as determined by PCA and correlation matrix (Fig. 5E,F). Thus, these results suggest that the cue for the recognition of the school odor is the mixing pattern of the PC molecular species that are blended for each school, but not any limited molecular species that is specialized for each school. In fact, modification of the PC profile, by addition of synthetic PCs, resulted in loss of the activity (Fig. 6). Thus, the complete PC profile is necessary for recognition of the school odor that underlies the organization of the *P. lineatus* school.

Use of chemical blends might increase the specificity of recognition and allow the transmission of more complex messages because the use of mixtures is well documented in many species and situations (Dulac and Torello, 2003). In ants, pheromones, often released as chemical blends, show extreme specificity in identifying the social status of the ants. Alarm pheromones are mostly composed of two or more chemicals that are used simultaneously to alert, attract and evoke aggression (Ono et al., 2003). In goldfish (a vertebrate), steroid hormone products have been identified as pre-ovulatory sex pheromones. Interestingly, three different pheromone blends can elicit varying degrees of male courtship or aggressive behavior in recipients (Poling et al., 2001). Group organization also requires specificity of recognition between group members in order to take advantage of many adaptive functions such as anti-predation, energy consumption, food location, migration and cooperative reproduction (Bradbury and Vehrencamp, 1998).

Chemical signals involved in the recognition of group members are, therefore, often constructed from the mixture of the same repertoire of chemicals. For instance, to recognize individuals social insects use a profile of cuticular hydrocarbons, whose constituents are different from nest to nest (Lahav et al., 1999; Ozaki et al., 2005; D'Ettorre and Heinze, 2005). Likewise, vertebrates also use chemical profiles as communication signals to discern the individual status of conspecifics such as parent–progeny, mating partner and familiarity (Kerth and König, 1999; Safi and Kerth, 2003; Bloss et al., 2002; Heymann, 2006). In the common marmoset, each female was found to have a unique ratio of highly volatile chemicals in the scent mark that could affect individual discrimination, which may play a key role in regulating both female intrasexual competition and intersexual communication as well as in providing a basis for the assessment of individual quality (Smith et al., 2001; Smith, 2006). These reports, however, only proposed candidates for chemicals underlying group identity and did not identify the chemicals. Our report is thus the first identification of a chemical signal underlying recognition of group odor in vertebrates. The mode of recognition of school odor mediated by the PC profile in *P. lineatus* resembles a chemical signature, enabling recognition

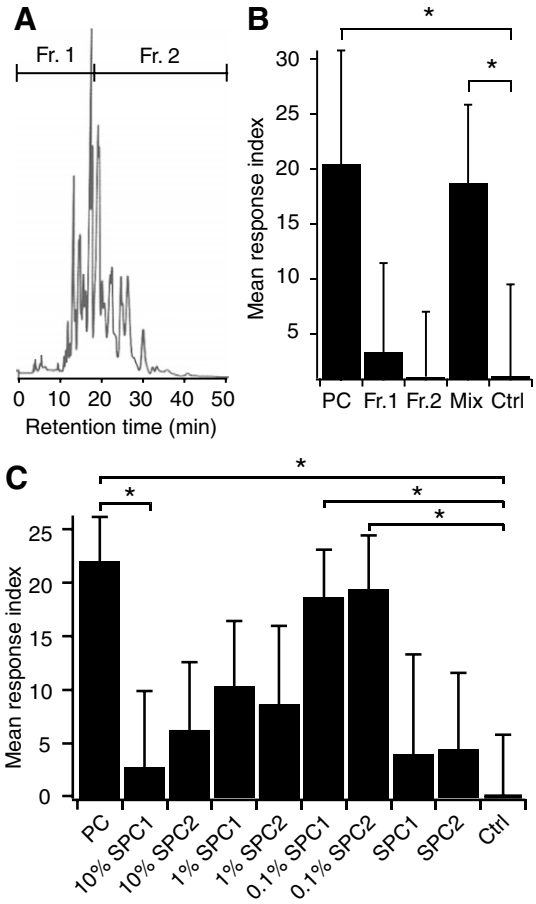

Fig. 6. Responses of *P. lineatus* to the PC profile. (A) HPLC separation of PC components into two fractions, Fr. 1 and Fr. 2. *x*-axis, retention time; *y*-axis, signal intensity. (B) Response of *P. lineatus* to Fr. 1, Fr. 2, and the recombined PC fraction (Mix). Values are means ± s.e.m. Asterisks, $P<0.05$. (C) Responses of *P. lineatus* to PC, the composition of which was modified by the addition of synthetic PCs SPC1 (16:0-22:6) [1-palmitoyl-2-(4,7,10,13,16,19-(E)-docosahexaenoyl)-sn-glycero-3-phosphocholine] and SPC2 (16:0-20:4) [1-hexadecanoyl-2-(5,8,11,14-(*E*)-eicosatetraenoyl)-sn-glycero-3-phosphocholine]. The PC activity was gradually affected depending on the concentration of SPC. Values are means ± s.e.m. Asterisks, $P<0.05$. Ctrl, control.

of the group, as described in other animals. The present work thus provides an important example of chemical profile-based recognition as a common scheme for group integration. The remaining issue is to identify which PC molecular species are essential for the expression of activity.

Influence of chemical profile

Communication *via* a chemical signal, as described above, is influenced by both genetic factors and environmental conditions. As a genetic factor, it is well known that the major histocompatibility complex (MHC)-related metabolites are involved in a variety of individual choice processes (Milinski et al., 2005; Willse et al., 2005; Boehm and Zufall, 2006). Although the mechanism for odor production influenced by MHC loci is not entirely clear, some rodents and humans are able to distinguish individuals who vary in this gene complex. In addition, mouse urinary proteins, members of the lipocalin family, are responsible for the binding and release of volatile chemicals, a contributing role in the communication of individual identity (Hurst et al., 2001). A similar mechanism might be involved in the case of *P. lineatus* if recognition of school members occurs among individuals that share similar MHC regions. Genetic similarity between school members could be explained from the beginning of *P. lineatus* schooling. During the spawning season, a spawning pair of *P. lineatus* digs a nest on a sandy bottom under rocks in shallow water where hundreds of eggs are laid. After the egg clutch has hatched, the juveniles stay on the bottom and start schooling within a week (Moriuchi and Dotsu, 1973). Therefore, school members must have a similar genetic composition as well as similar MHC loci. However, genetic similarity of school members becomes more unpredictable as they grow, because fully grown schools will have already been mixed repeatedly with other schools. Accordingly, the degree of genetic relatedness among school members must be a key to understanding school organization in *P. lineatus.*

In addition to genetic factors, environmental factors also influence the composition of signal substances, with diet being particularly important. In fact, diet manipulation changes body odors that are involved in individual recognition and social relationships in various animals (Bryant and Atema, 1987; Liang and Silverman, 2000; Olsén et al., 2003). This implies that diet is a significant factor in the production of a distinctive body odor. Similarly, the diet condition of a *P. lineatus* school might contribute to the diverse heterogeneity of PC profiles between schools, as shown in Fig. 5A–E. Therefore, in addition to the examination of genetic similarity, PC molecular species collected from schools at every growth stage must be analyzed. In other words, the results obtained could provide a possible mechanism for integrating school organization of *P. lineatus* from the aspects of both genetic factors and environmental conditions.

Perception of chemical mixture

Odor mixtures and their patterns can elicit characteristic responses at every stage of sensory processing: olfactory receptor (Oka et al., 2004), olfactory bulb (Tabor et al., 2004), olfactory cortex (Zou and Buck, 2006), and behavioral expression (Valentinčič et al., 2000; Uchida and Mainen, 2003). Recently, the neural response to natural odors such as seasoning, food and animal odors was elegantly recorded from the dorsal surface of the main olfactory bulb, indicating that odor mixtures are encoded by sparse representation in the olfactory bulb (Lin et al., 2006). These learning and processing mechanisms for complex odor mixtures can help us to understand the perception mechanism of PC molecular species in *P. lineatus*. For example, *P. lineatus* starts schooling soon after hatching from an egg clutch (Moriuchi and Dotsu, 1973), suggesting that the familiar school-specific odor (PC profile) is imprinted during the early developmental stage. In addition, *P. lineatus* forgets the familiar school odor within a day, and a lost individual can learn an unfamiliar school odor (Kinoshita,

1975). The results indicate that school recognition in *P. lineatus* is plastically controlled after growth. Therefore, the learning mechanism of school-specific odor (mixing pattern of PC molecular species) could be an important example in which to investigate the rewiring and maintenance of neural substrates underlying the memory of complex chemical mixtures.

Our study of the complex chemical mixture underlying the recognition of group odor by *P. lineatus* provides valuable insights into recognition mechanisms mediated by chemical signals. Furthermore, understanding how such a complex signal is represented will not only provide insight into neural strategies for coding of social signals, but also suggest a paradigm for understanding more generally how natural olfactory scents are represented in the olfactory bulb, and beyond that, how social behavior is conducted.

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