Preen Gland-Secreted Alkanols Enhance Male Attractiveness in Parrots

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Chemical communication is widely used throughout the animal world including birds (1-3). The skin glands of vertebrates can release chemical signals such as sex pheromones attracting the opposite sex for breeding (1,2,4). But, the uropygial (also called preen or oil) glands of birds have no sex pheromones characterized (2,3,5). Here, we show that females of the budgerigar, *Melopsittacus undulatus*, can distinguish males from females via body odour in Y maze, indicating an occurrence of sexual dimorphic odours. We thus hypothesize that the uropygial gland secretions preened into body plumage contribute to the attracting body odorants. Chemical assay reveals that the gland-producing volatile octadecanol, nonadecanol and eicosanol have fourfold higher relative abundances in males than in females and were regarded male pheromone candidates as in rodents(4,6,7). Meanwhile, females show preferences for the odor of one-male-plumage equivalent of the secretion or the alkanol blend over the female counterparts; so do females for a male sealed inside a transparent jar scented outside with the male alkanol blend over the other male inside a jar with the lower-dose blend. This suggests that the male alkanol blend is a sex pheromone and the gland has broader implications than previously known in sexual behaviour of birds(8).

The budgerigar (*Melopsittacus undulatus*), a small parrot native to Australia, living in flocks and the most popular cagebird worldwide, is known using vocal behavior, plumage colouration, fluorescent and ultraviolet light for sexual attractiveness (8-11).Whereas sexual dimorphism of colouration and vocalization looks less remarkable in budgerigars than in some songbirds. Here, we asked whether chemical signals contribute to gender discrimination in the parrots. Although chemical communication in birds remains largely unknown compared to other animals, some species of birds have shown the use of such a communication modality when using self-produced odour to regulate social behavior (3). For example, the Antarctic prion (*Pachiptila desolata*) can recognize its partner by individual odours (12), and the crested auklet (*Aethia cristatella*) is attracted by conspecific feather odours (*cis*-4-decenal and octanal) during courting (13).

Birds indeed have olfactory perception capacity and self-produced odours comparable with mammals (2,12-22). Thus, chemical communication must be more widely used by birds than previously known (2,3). Therefore, like in mammals, serving sex recognition of the first step of breeding behaviour, sex-attractant pheromones, which are chemical signals released by animals to attract opposite sex conspecifics, are supposed to exist in birds (1,2). Using Y-maze test, we found that female budgerigars spent more time investigating the choice arm with either living male demonstrator ($Z=2.701$, $N=16$, $P=0.007$, Wilcoxon matched-pairs test unless otherwise noted) or stream flowed through a stimulus jar with a male bird sealed inside ($Z=2.298$, $N=16$, $P=0.025$) than the other arm with the counterparts of a female (Fig. 1a), suggesting that male budgerigars did emit some volatile substances distinct from females' to evoke responsiveness of females, or that females had the olfactory ability to discriminate males from females.

The specialized skin glands such as mammalian sebaceous glands can produce pheromones in addition to other substances (e.g. oily wax esters) (1,2). Serving as only skin gland in birds, the uropygial gland, also called preen gland, oil gland or scent gland, has been focused chemically on its nonvolatile wax compositions and functionally on its association with light reflectance and waterproofing quality of the plumage (5,8,11). Growing evidences show that the gland produces some low-molecular-weight volatiles, which are associated with species, gonadal conditions and gender in birds just as some structurally similar pheromone compounds of insects and rodents do. (1,4,7,19-21,23,24). This indicates that the gland is a possible pheromone-prodcucing source of birds. Indeed, birds can beak the glandular secretions to the body plumage when preening, resulting in a possible transmission of the chemical signals via plumage.

Using GC-MS (gas chromatography-mass spectrometry) analysis, we characterized the peaks early-eluting from the capillary GC (before 40 min) as hexadecanoic acid, heptadecanol, octadecanol (18OH), nonadecanol(19OH), eicosanol (20OH), heneicosanol and 15 pentanoates with linear alcanol or alcenol (C_{16}-C_{20}), and the late-eluting peaks (after 40 min on chromatogram) as ester waxes with Long-chain acids (C_{16}-C_{18}) (Fig. 2 and S1). The early-eluting compounds have the similar chemical properties to those of the pheromone compounds produced by insects and rodents and were therefore subjected to pheromone candidate search (1,4,23,24,). Some linear alkanols(C_{10}-C_{18}) are major volatile components of the preen glands of the dark-eyed Junco(*Junco hyemalis*) and the Bengalese finch, too (*Lonchura striata*) (20) (J.-X.Z., L., Sun and M.-X. Zuo, unpublished data).

Male pheromones are usually among the skin gland-secreted volatiles that are male-specific or higher in concentration or relative concentration in males than in females(4,6,7,24,25). In the parrots, neither male-specific volatiles nor those having higher contents (reflected by GC peak area) in males than in females were detected from the preen glands, despite females having more abundant pentanoates (Fig. 2;Table S1). The glands did not show sexual dimorphism in size and consequent secretion production yet (Data not shown). The further comparison of percent GC peak areas revealed ultimately that the glandular hexadecanoic acid and alkanols had significantly higher relative concentrations in males than in females in budgerigars ($N=8$, $P<0.05$, independent t test) (Table S1). These volatiles were regarded male pheromone candidates. In particular, 18OH, 19OH and 20OH had fourfold higher relative concentrations in males than in females ($N=8$, $P<0.01$ for 18OH, $P<0.001$ for 19OH and 20OH, independent t test) and were equivalent to approximately 73% of all the volatiles of males (table S1), we thus focused our further pheromone searches on the three prevailing odourants (4, 6). Meanwhile, the contents of 18OH, 19OH and 20OH determined by GC-MS were 3.58±3.06 (n=8) μg, 2.78±2.67 (n=8) μg and 5.32±3.10μg (n=8) per mg of the glandular secretion, respectively.

Using dichloromethane extraction, we revealed that the uropygial gland secretion and body plumage of budgerigars, like in other birds, had quite similar GC profiles, particularly in the fractions of late-eluted wax esters (Fig 2, S1, S2 and S3)(20); using headspace sampling, we detected 18OH, 19OH and 20OH from the body odours (Fig. S4). This suggested that the secretion was transferred from the gland into the body plumage and consequently gave off the gases of the alkanols in the budgerigar. Some GC peaks were additionally increased or created by likely feather-originated compounds such as hexadecanoic, octadecenoic, octadecanoic acids and squalene (Fig. S2, S3 and S4).

The amounts of 18OH preened into the body plumage was first determined 8.38±4.47 (n=8) ηg per mg of feather via dichloromethane extraction. Then, 19OH and 20OH were calculated 6.51ηg and 12.45ηg per mg of feather on average, respectively, according to the ratio (w/w) of them to 18OH in the glandular secretion. Then, each bird roughly had 34 μg 18OH, 26 μg 19OH and 50

μg 20OH, being equivalent to those in about 10 mg glandular secretion, spreaded over the whole body plumage (presumable 4 g for each bird), according to which the synthetic analogs were blended and used as one-male plumage equivalent for presentation in bioassay of their attractiveness to females.

To validate the pheromonal activity, the above-mentioned alkanol blend was applied in 40 μl dichloromethane to a Petri dish (inner diameter:6 cm) and fitted to the stimulus jar connected to the Y maze. Similarly, a low-dose alkanol blend (8 μg 18OH, 6.5 μg 19OH and 12.5 μg 20OH), presumably reflecting their low ratios in females, was prepared according to the sexual differences in relative concentrations and used to contrast with the males'. In Y maze, female budgerigars spend more time investigating the arm with the stream flowed through either a crude dichloromethane extract of 10 mg glandular secretion ($Z=2.639$, $N=16$, $P=0.008$) or the high-dose alkanol blend ($t=2.236$, $N=16$, $P=0.041$, paired t-test) of males than the other arm with the counterparts of females, suggesting that the glandular secretion itself or the blend of its 18OH, 19OH and 20OH convey air-borne information about maleness (Fig 1.b). In addition, the blend no longer functioned in the sexual arousals upon removing any alkanol out of it (data not shown). Thus, the male alkanol blend was a sex-attractant pheromone of budgerigars.

Additional experiments were performed to illuminate the functional significance of chemical communication modality. We showed that caged females chose the male sealed inside a transparent jar close the cage over the female inside another jar at the opposite side ($Z=2.501$, $N=16$, $P=0.012$,), suggesting that females were capable of discriminating a male from a female, whose odours were screened by the jar, in light of the visual and weakened acoustic signals, as previously reported, in such a two-choice device (8-11) (Fig.3, Fig. S5).So did females a male inside a jar attached by an outside Petri dish scented by the alkanol blend of males than the other male inside a jar with the low-dose blend of females ($Z=2.555$, $N=14$, $P=0.011$), indicating that pheromones did enhance male attractiveness (Fig. 3;S5). While, females preferred the jar with a male bird, free of the blend, to the other jar scented outside with the high-dose blend, free of birds($Z=2.111$, $N=16$, $P=0.035$), implying that physical signals evoked stronger responsiveness from females than the chemical signals did (Fig.3b). As a result, olfaction involvement in bird communication could increase the precision of gender recognition and sex selection in combination with vision and audition. Alternatively, olfaction may become prominent in sexual communication in inaudible (e.g. noisy) and invisible (e.g. dark) surroundings (18).

Here, we demonstrated a multicomponent sex-attractant pheromone derived from the uropygial gland of birds. The pheromone functioned in sexual attractiveness, like those of rodents, relying on the sexual variation of the relative abundance of its components (4,6,7). Our findings suggested that uropygial glands and preening behaviour had broader implications than previously believed (e.g. plumage reflectance and protection) in gender recognition and sex selection of birds (8,11).

Method summary

Twenty-four pairs of adult wild-typed budgerigars were from pet suppliers. We pressed living birds' glands to lead the secretion outside. 1-mg secretion or wing feather was extracted in 20 μl dichloromethane. Purified air stream was flowed through a jar with a bird and directed to a Porapak-Q trap to sample body odor. GC-MS (Agilent 6890N GC, 5973 Mass Detector, NIST 2002) was used. The GC had a HP5-MS column (30m×0.25mm i.d.×0.25-μm film thickness), carrier gas Helium at 1.0ml/min and injector at 280°C. The oven was programmed at 5°C/min from 70°C up to 280°C. MS was in Electron impact mode (70 eV). The candidate compounds were identified from NIST2000 and commercial authentic

analogs. The relative concentration was quantified by percentage of each of the 23 volatile GC peak area. The contents of akanols in samples were determined by comparing their GC areas with the standard curve or calculated. The Petri dish was scented by stimulus odourants for presentation. Each choice arm of Y-maze has a rear demonstrator compartment or a steam flowed through an odour stimulus jar. The test-cage consisted of a choice cage and a start cage. Each trial lasted for 3 min and recorded by videotapes.

Fig. 1: **Behavioural responses of female budgerigars (N=16), *M. undulatus*, in Y maze, (a),** to male (M) and female (F) birds or their body odour (N=8 for each sex) (**b),** to uropygial gland secretion (UPGS) or blends (P) of octadecanol, nonadecanol and eicosanol of males and females. (Wilcoxon matched-pairs test was used, *p<0.05; **P<0.01).

Fig. 2: Volatile fractions of 23 to 40 min of retention time on representative GC chromatograms of uropyginal gland secretion of males (RED) and females (GREEN) (GC Peaks1,2,3,4,6 and 9 refer to hexadecanoic acid and heptadecanol, octadecanol, nonadecanol,eicosanol and heneicosanol, repectively; peaks 5,7,8 and 10 to 23 refer to pentanoates with characteristic ions at m/z 85 and 103 and linear alcanol or alcenol chains (C_{16}-C_{20}).

Fig.3: Behavioural responses of female budgerigars (N=16), *M. undulatus*, in a test cage, beside which are two jars to seal demonstrated birds inside(N=8), and under the bottom of the cage are two Petri dish scented by the blends of octadecanol, nonadecanol and eicosanol for presentation.
(MB=male birds; FM=female birds; MP= the high-dose alkanol blends as in males; FP= the low-dose blends as in females).

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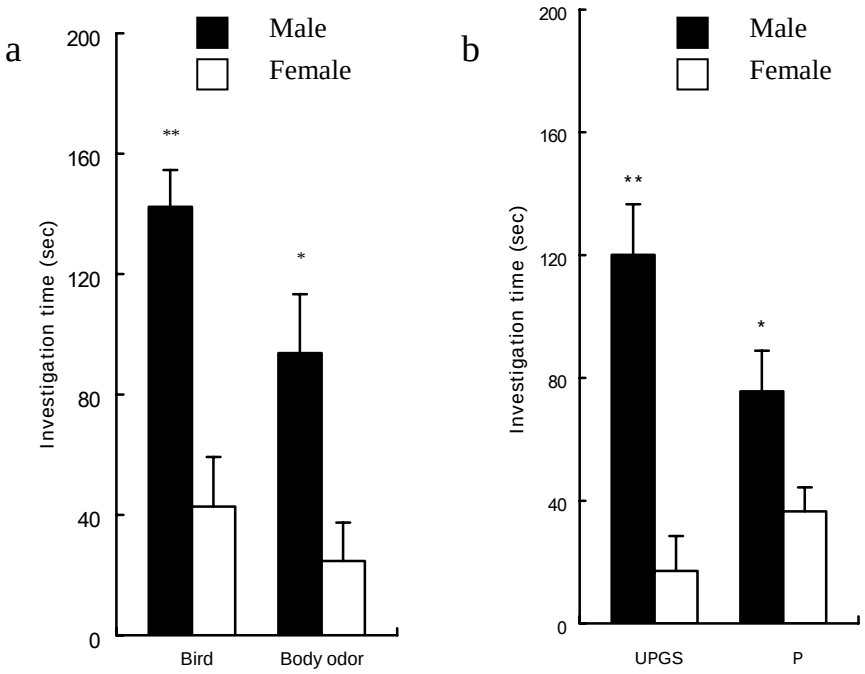
a
Male
Female
Investigation time (sec)
200
160
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80
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Bird
Body odor
b
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Investigation time (sec)
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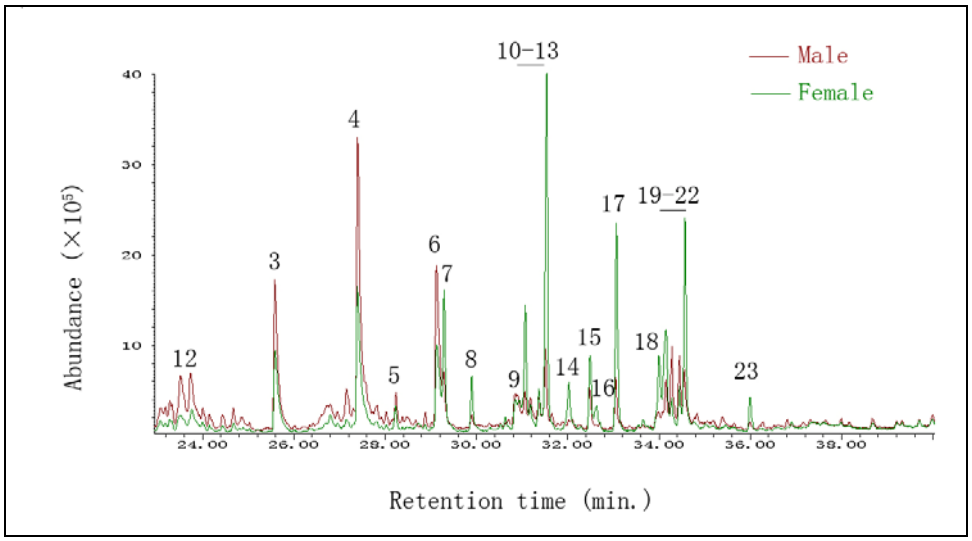
UPGS
P

Fig. 1


Male
Female
Abundance (×10⁵)
40
30
20
10
10–13
4
3
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7
17
19–22
12
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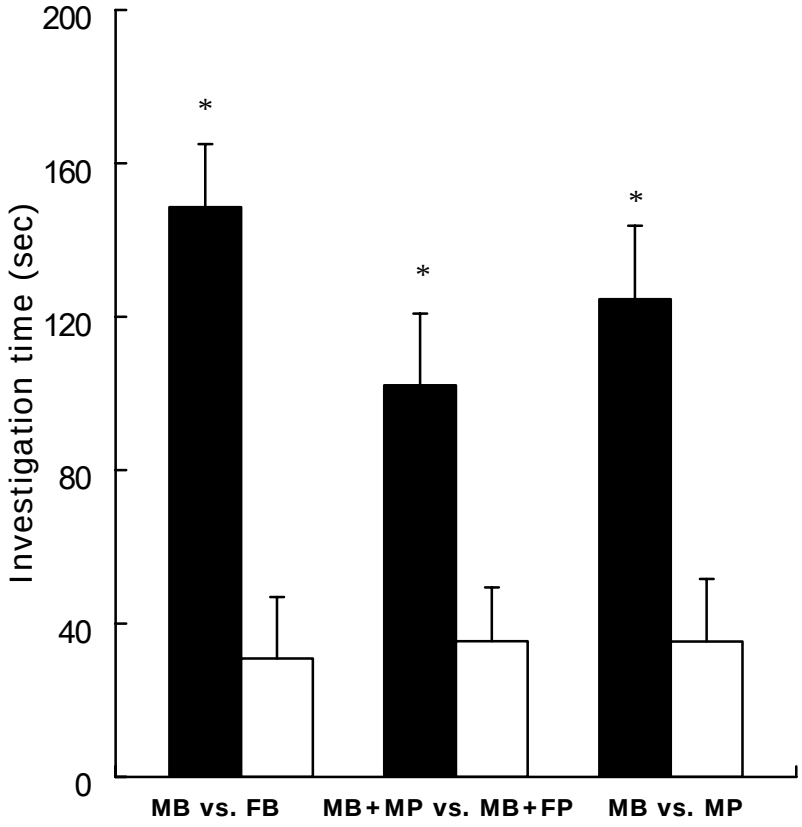
34.00
36.00
38.00
Retention time (min.)

Fig. 2


200
160
120
80
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Investigation time (sec)
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MB vs. FB
MB+MP vs. MB+FP
MB vs. MP

Fig. 3: