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Red junglefowl have individual body odors

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SUMMARY

Olfaction may play an important role in regulating bird behavior, and has been suggested to be involved in feather-pecking. We investigated possible differences in the body odors of red junglefowl females by using an automated olfactometer which assessed the ability of trained mice to discriminate between the odors of uropygial gland secretions (the main carrier of potential individual odors in chickens) of six feather-pecked and six non-pecked birds. All mice were clearly able to discriminate between all individual red junglefowl odors, showing that each bird has an individual body odor. We analyzed whether it was more difficult to discriminate between the odors of two feather-pecked, or two non-pecked birds, than it was to discriminate between the odors of two randomly selected birds. This was not the case, suggesting that feather-pecked birds did not share a common odor signature. Analyses using gas chromatography and mass spectrometry showed that the composition of aliphatic carboxylic acids in uropygial gland secretions differed consistently between individuals. However, chemical composition did not vary according to feather-pecking status. We conclude that red junglefowl have individual body odors which appear to be largely based on differences in the relative abundance of aliphatic carboxylic acids, but there is no evidence of systematic differences between the body odors of pecked and non-pecked birds.

Key words: red junglefowl, body odors, mice, GC–MS, feather-pecking.

INTRODUCTION

Evidence from a diverse range of animals indicates that body odors play an important role in recognition, communication and behavior. Body-borne chemical signals are used for kin recognition and mate choice, but also for individual identification of conspecifics (Eisenberg and Kleiman, 1972; Wyatt, 2003). Recently, based on gas chromatography analysis of uropygial gland secretions, it was suggested that feather-pecking, a detrimental behavior disorder in egg-laying hens, could involve olfactory discrimination of potential victims in a flock (Sandilands et al., 2004). However, so far the extent to which individual chickens actually differ in their body odor, or how such differences would be related to the status of being feather-pecked has not been examined.

Mammals are well known for using olfactory social communication whereas birds are traditionally considered to be microsmatic and to rely on sight and sound rather than olfaction (reviewed by Balthazart and Taziaux, 2009; Hagelin and Jones, 2007; Jones and Roper, 1997). However, a growing body of evidence suggests that olfaction may play an important and hitherto underestimated role for birds in different contexts (Balthazart and Taziaux, 2009). A series of studies has shown that seabirds strongly rely on their sense of smell. Antarctic prions (*Pachiptila desolata*) were found to be able to recognize and discriminate odors of individual conspecifics (Bonadonna and Nevitt, 2004) and blue petrels (*Halobaena caerulea*) have been shown to rely on odor cues to recognize their own burrows (Bonadonna et al., 2004). Moreover, several studies have shown that chickens respond to olfactory cues (Jones and Gentle, 1985; Jones and Carmichael, 1999; Jones et al., 2002; Mabayo et al., 1996) and that they prefer the odors of familiar soiled substrate over odors from unfamiliar or clean substrate (Jones and Gentle, 1985). A study by Burne and Rogers suggests that chickens learn about their olfactory environment during the later part of incubation and in the early post-hatching period (Burne and Rogers, 1999). Furthermore, the chicken genome contains at least 229 genes coding for olfactory receptors (Lagerström et al., 2006) and physiological studies have shown that chicken olfactory neurons display properties similar to those found in other vertebrates (Jung et al., 2005). These findings suggest that the role of olfaction in bird social behavior may have been underestimated, and call for more research in this area.

Exudates from cutaneous glands in the skin contribute to individual body odor and olfactory communication in mammals (Eisenberg and Kleiman, 1972). Bird skin is devoid of glands except for the uropygial gland, located at the base of the large tail feathers of birds. This gland produces preen oil which the bird spreads over its body with its beak during preening (Schmidt et al., 2003). The chemical profile of individual petrels is consistently similar from year to year, and different from that of other birds, suggesting the existence of an endogenous individual olfactory signature in this species (Bonadonna et al., 2007).

In chickens, uropygial gland secretions and feather extracts have been analyzed with gas chromatography–mass spectrometry (GC–MS) (Sandilands et al., 2004). Carboxylic acid composition was found to be affected by age, lipid source and whether the bird had been exposed to feather pecking, a detrimental behavior disorder among chickens. A clear difference was evident in the relative proportions of carboxylic acids, leading the authors to predict that this difference may affect the plumage odor and therefore its attractiveness to other birds as a pecking inducer.

Based on earlier findings showing that carboxylic acid composition differs among chickens (Sandilands et al., 2004), the aim of the present study was to investigate whether this is also

reflected in individual body odors and to assess whether there are similarities in odor among animals exposed to feather pecking. Red junglefowl is the common ancestor of all domesticated chickens, and therefore could be said to represent 'the origin' of their behavior and physiology (Siegel et al., 1992; West and Zhou, 1989). Rather than relying on chemical analysis only, we used an automated olfactometer which assessed the ability of four trained mice to discriminate between the odors of uropygial gland secretions from individual birds that were either pecked or non-pecked. Furthermore, the body odors of the chickens were investigated by GC–MS analysis of uropygial gland secretions, in order to compare the chemical composition of the stimuli with the discrimination ability of the mice.

MATERIALS AND METHODS

Animals

The chickens used for odor sampling were 2 year old red junglefowl (*Gallus gallus* L.) females kept at the Wood-Gush chicken research house at Linköping University, Sweden. The population stems from a Swedish zoo population that was brought from Thailand for breeding (Schütz and Jensen, 2001). The animals were kept in the facility for the purpose of a breeding program as part of ongoing research in behavior genetics. Full details of animal housing and husbandry systems are given elsewhere (Campler et al., 2009). Briefly, the animals were kept in sex-separated groups of about 30–40 birds each in a 3 m×4 m pen with elevated perches, nest boxes and combined feeding and dunging shelves. Feather pecking was common, and within a group the plumage status of the animals varied from severely pecked, with large naked areas, to not pecked at all. Six severely feather-pecked birds (P) and six non-pecked birds (NP) were identified by visual inspection of plumage condition on the back of the chicken. From each bird, fresh samples of uropygial gland secretion were obtained for subsequent olfactory discrimination testing (see below for details on sample collection).

Testing was carried out using four male CD-1 mice (*Mus musculus* L.), an outbred mouse strain more similar to wild-type mice than inbred laboratory strains. Mice are known for their excellent olfactory discrimination abilities and the mice used in this study were already trained to operate the olfactometer and discriminate between different odors (Laska et al., 2007; Laska et al., 2006). Animals were housed individually in standard plastic cages in a temperature- and humidity-controlled room and maintained under a 12 h:12 h light/dark regime. The mice were approximately 120 days old at the beginning of the study. During the experiments, the animals were kept on a water-deprivation schedule of 1.0 ml of water per day. The experiments reported here comply with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 86-23, revised 1985) and were performed according to a protocol approved by the local Ethical Committee of The Swedish National Board for Laboratory Animals.

Odor stimuli

Secretion samples from the uropygial gland of red junglefowl were used as stimuli. Birds chosen for sampling were isolated from their home groups and housed in individual furnished laying hen cages, containing perches, a dust bath and a nest. The birds were in full visual and acoustic contact with other birds during the sampling period. Uropygial gland secretion samples were collected by gently squeezing the area around the uropygial gland. Extracted oil droplets were sampled on Pur-Zellin® pads (Hartmann-ScandiCare AB, Anderstorp, Sweden) and immediately put in sealed plastic bags to prevent contamination. Fresh samples were taken before each test session.

Behavioral test

Olfactory discrimination ability was assessed using an automated olfactometer (Knosys, Tampa, FL, USA). Mice were trained using standard operant conditioning procedures (Bodyak and Slotnick, 1999) to insert their nose into the odor sampling port of a test chamber. This triggered a 2 s presentation of either an odor used as the rewarded stimulus (S+) or a different odor used as the unrewarded stimulus (S–). Licking at a steel tube providing 2.5 µl of water reinforcement in response to presentation of the S+ served as the operant response. Accordingly, not licking in response to presentation of the S– was regarded as a correct rejection. One-hundred such trials (50 S+ and 50 S– trials in pseudorandomized order) of a given pair of S+ and S– were conducted per animal and condition.

Two mice were trained with a P odor as the rewarded stimulus (S+) and the other two with an NP odor as the rewarded stimulus (S+). Each S+ was tested against several different S– of both P and NP odors. As a control the familiar S+ was exchanged for a novel S+ on three occasions. A complete list of the stimulus combinations tested is given in Table 1.

The tests were preceded by a training process in which the mice were presented with uropygial gland secretion samples as S+ stimuli *versus* a blank sample pad as the S– stimulus. From this the mice learned to discriminate the uropygial gland secretion odors from the odors of the pad. This training process continued until all mice scored above 85% correct choices, implying that they had learnt to correctly assign the reward value of the S+. For every new S+ the mice went through a new training process.

GC–MS analysis

Samples were taken from all animals on two separate occasions with a 4 week interval. The sampling was done using the procedure described above. The uropygial gland secretion was diluted with CH_2Cl_2 (20 µl) and stored at –18°C until analyzed. A derivatization solution consisting of BF_3OEt_2 (5 ml, 82.58 mmol) in MeOH (15 ml) was prepared and used for all analysis. All solvents were of analytical grade (Merck, Darmstadt, Germany).

GC–MS analyses were performed using an HP 6890 gas chromatograph with an HP 5973 mass selective detector. The GC was run in split-mode with a head pressure of 10 p.s.i. and a helium flow rate of 1.2 ml min^{-1}. The mass detector was run in the electron impact (EI) mode using an ionization voltage of 70 eV and a source temperature of 230°C with a constant GC injector temperature at 250°C. The ions were scanned in the total ion current (TIC) or in

Table 1. Stimulus combinations tested with the four mice

Mice 1 and 2		Mice 3 and 4	
S+	S–	S+	S
NP1	P2	P1	N2
NP1	P3	P1	N3
NP1	P4	P1	N4
NP5	P5	P5	N5
NP1	NP2	P1	P2
NP1	NP3	P1	P3
NP1	NP4	P1	P4
NP5	NP6	P5	P6
P1	P6	N1	N6

S+ refers to the rewarded stimulus and S– to the unrewarded stimulus in a given combination. NP1–6 refers to uropygial gland secretions from six individual non-pecked birds, and P1–6 refers to uropygial gland secretions from six individual pecked birds.

the selected ion monitoring (SIM) mode. The chromatographic separation was carried out on a FactorFour^{TM} capillary column (Varian, VF-5 ms, 30 m×0.25 mm×0.25 μm. The separation was performed using a temperature program consisting of an initial hold at 120°C for 3.0 min then ramp to 160°C at a rate of 15°C min^{-1} then ramp to 250°C at a rate of 10°C min^{-1} and held at 250°C for 20.0 min. The injection volume was 2 μl and a split mode at 30:1 was chosen.

A mixture of the sample, diluted with $CHCl_3$ (0.2 ml) and the derivatization reagent (1 ml) was added to a sealed tube and heated to 90°C. After 1 h, the reaction was quenched with H_2O (0.9 ml) and extracted with *n*-pentane (0.6 ml). The organic phase was concentrated under a stream of nitrogen and the residue was dissolved in $CHCl_3$ (30 μl) and further used for injection.

Data analysis

For each individual animal, the percentage of correct choices from 100 decisions per stimulus pair was calculated. Additionally, the percentage of correct decisions in the first block of 20 trials per task (comprising 10 S+ and 10 S– trials in pseudorandomized order), and in correct rejections of the S– in the first block of 20 trials per task was analyzed. This was done as previous studies have shown that differences in task difficulty may require a more detailed analysis than provided by the percentages of correct decisions from all 100 decisions collected per task and animal (Laska et al., 2008; Laska and Shepherd, 2007).

Significance levels were determined by calculating binomial *z*-scores corrected for continuity from the number of correct and false responses for each individual and condition. Comparisons across tasks were made using Friedman's two-way analysis of variance. When ANOVA detected differences between tasks, this was then followed by pairwise Wilcoxon's signed-rank tests for related samples to evaluate which tasks were responsible for the difference. All tests were two-tailed and the α-level was set at 0.05.

The gas chromatographic profiles of the uropygial gland secretions were treated as frequency distributions of different carboxylic acids. Therefore, Spearman rank-correlation tests were used to assess differences between samples in these profiles. More specifically, we compared samples taken from the same animal on different days (intraindividual comparisons) as well as all possible combinations of samples taken from different animals (interindividual comparisons). This included comparisons between samples of pecked and non-pecked birds as well as comparisons of individuals within each of these groups. If the chromatographic profiles were more similar within the P and NP groups than between birds from different groups, the median r_s would be higher within than between groups. This was examined by calculating the median r_s values from the Spearman tests, and comparing those between P and NP birds using Mann–Whitney *U*-tests for independent samples. To further examine possible within-group similarities, the chromatographic profiles of each individual (the mean of the two individual samples) were entered into a hierarchical single-link cluster analysis. If the profiles were more similar within than between groups, we expected samples to cluster primarily within groups.

RESULTS

General discrimination performance

Fig. 1 shows the performance of the mice in discriminating between various combinations of uropygial gland secretions. Considering the mean percentage of correct decisions across all 5 blocks of 20 trials performed per animal and task (Fig. 1), all four mice performed significantly above chance level (>85% correct decisions) in all nine tasks and thus were clearly able to discriminate between all combinations presented (two-tailed binomial test, $P<0.01$ with all stimulus combinations and animals). Considering the performance in only the first block of 20 trials performed per animal and task (Fig. 1, squares) the mice showed quick learning (>70% correct decisions) in all nine tasks. Because of the good discrimination ability we must take into consideration the performance of the mice in the learning phase where the animals are challenged with the difficult part of learning to reject the S–. The mean percentage of correct rejections in the first block of 20 trials performed per animal and task ranged from 47.5% to 82.5% (Fig. 1, triangles) suggesting that some discrimination tasks were slightly more difficult than others.

Discrimination of odors from pecked and non-pecked chickens

Fig. 2 shows the mean performance of all four mice when the first four sets of data points and the remaining five sets shown in Fig. 1 are combined. The data points on the left describe the average performance of the mice across the four tasks in which they were presented with NP odors as S+ and with P odors as S– and *vice versa*. The data points on the right describe the average performance of the mice across the five tasks in which they were presented with NP odors as both S+ and S– or with P odors as both S+ and S–. Comparing the performance between these two groups of discrimination tasks yielded no significant difference (Wilcoxon, $P>0.05$).

GC–MS analysis

Fig. 3A shows a representative example of a gas chromatographic profile of the uropygial gland secretion of a red junglefowl.

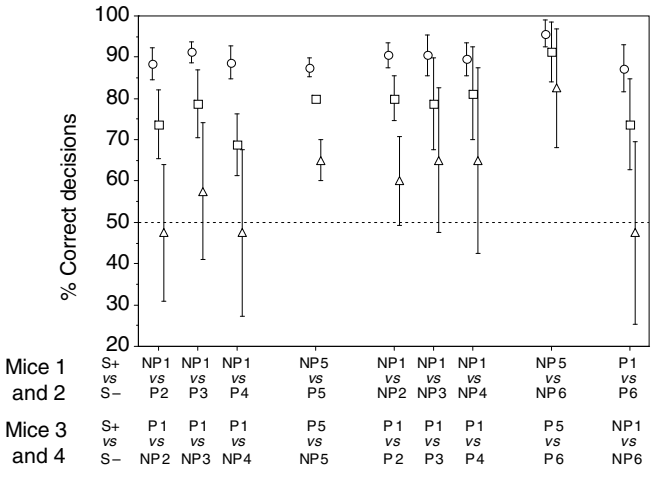

Fig. 1. Performance of CD-1 mice in discriminating between various combinations of uropygial gland secretions. Each data point represents the percentage (means ± s.d. from *N*=4 animals) of correct decisions per odorant pair (i) across the five blocks of 20 trials performed per animal and task (circles), (ii) in the first block of 20 trials (squares), and (iii) in correct rejections of the unrewarded stimulus (S–) in the first block of 20 trials (triangles). The four sets of data points to the left describe the performance of the mice across the four tasks in which they had to discriminate between P and NP odors and the five sets of data points on the right describe their performance across the five tasks in which they had to discriminate between two different P odors or two different NP odors. P1–6 and NP1–6 refer to odor samples from six pecked (P) and six non-pecked (NP) birds, respectively. The dashed line indicates the chance level of performance.

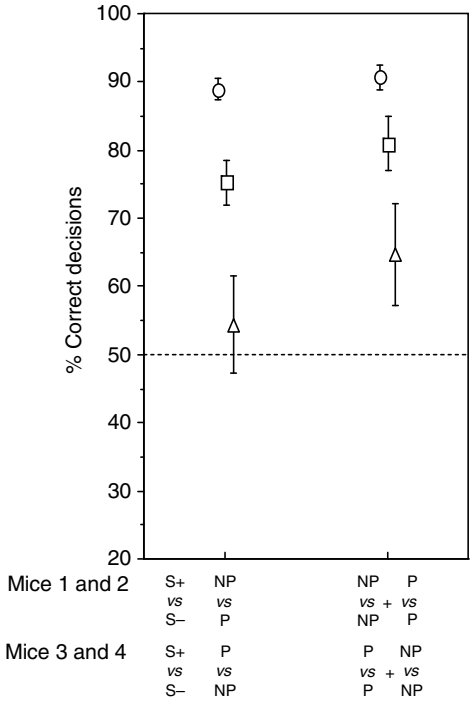

Fig. 2. Mean performance of all four mice when the first four sets of data points and the remaining five sets shown in Fig. 1 are combined. Data points on the left describe the performance of the mice across the four tasks in which they had to discriminate between P and NP odors and data points on the right describe their performance across the five tasks in which they had to discriminate between two different P odors or two different NP odors (indicated by the + sign between the two combinations). Symbols as for Fig. 1.

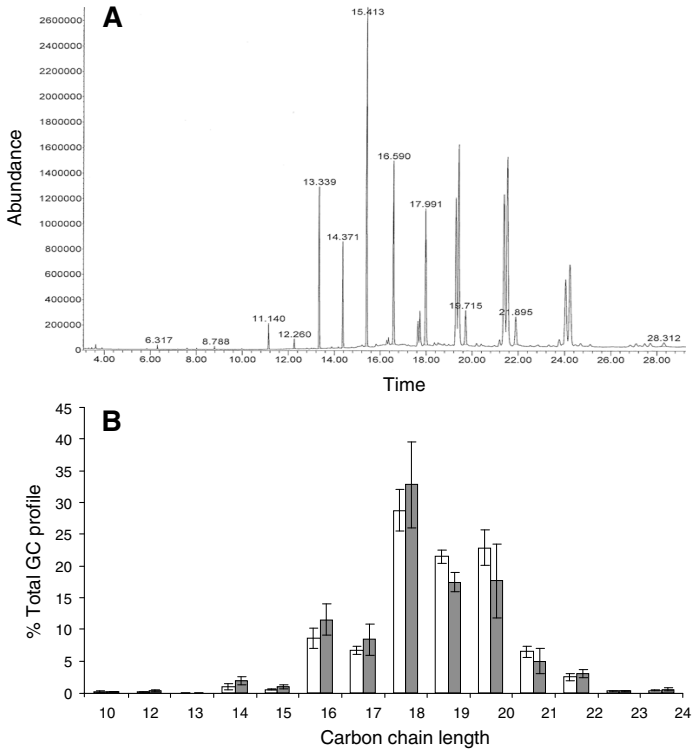

Fig. 3. (A) A representative example of a gas chromatographic profile of the uropygial gland secretion of a red junglefowl (time in minutes). (B) The relative abundance (means ± s.d.) of aliphatic carboxylic acids from pecked chickens (white bars) and non-pecked chickens (shaded bars).

Subsequent mass spectrometric analysis of the gas chromatographic effluent demonstrated that the secretions are mainly composed of aliphatic carboxylic acids with carbon chain lengths of C_{10} to C_{24}. Comparisons between samples taken from the same animal on separate days showed a highly significant correlation in the relative abundance of the carboxylic acids (Spearman, mean ± s.e.m. r_s=0.97±0.01; P<0.001 with all individual birds tested) indicating that the odor signature of a given individual bird was virtually identical across the 4 week sampling period.

The r_s values obtained from intraindividual comparisons of the relative abundance of the carboxylic acids were significantly higher than the r_s values obtained from the corresponding interindividual comparisons (Spearman, mean ± s.e.m. r_s=0.97±0.01 for intraindividual comparisons and r_s=0.93±0.01 for interindividual comparisons; difference between intraindividual and interindividual comparisons, Mann–Whitney, P<0.01). This suggests that individuals have consistent and individually different chromatographic profiles.

Comparisons between samples of pecked and non-pecked birds (Fig. 3B) showed some apparent variation in the relative abundance of the carboxylic acids between the two groups but not in the total amount of carboxylic acids. However, this was not significant, as the mean r_s within groups did not differ significantly from the mean r_s between groups (mean ± s.e.m. r_s=0.93±0.01 *versus* r_s=0.93±0.01; Mann–Whitney, P>0.05). The cluster analysis further showed that samples consistently clustered primarily between groups, indicating that there was low within-group similarity in the chromatographic profiles (Fig. 4).

DISCUSSION

The results of this study demonstrate that red junglefowl have individual body odors, which can easily be discriminated by trained mice (see Fig. 1). This ability is likely to be based on differences in the relative abundance of carboxylic acids in the individual uropygial gland secretions. However, we did not find any indication either from the discrimination ability of the mice or from gas chromatography analysis of the individual samples that feather-pecked birds would have any common olfactory signature which would allow them to be discriminated as a group based on odor (see Figs 2–4).

One might argue that the uropygial gland secretion may represent a limited aspect of the body odor of a chicken. However, chickens have few other integumental glands (Stettenheim, 2000), and initial pilot trials using swabs of the entire plumage showed that this produced very little odor (as judged by the ability of the mice to perceive the odor of such samples), and that the samples were easily contaminated by dirt in the feathers of the sampled birds. Another potential problem could be that the concentrations of the presented odors were different between different samples, depending on the amount of secretion obtained in a specific sample, but it is well established that mice are poor at discriminating between odor intensities compared with odor quality (Laska et al., 2008). Hence, we believe that our samples were truly representative of the body odors of the individual birds.

Another important limitation in our results is of course the fact that we have used mice for odor detection and discrimination rather

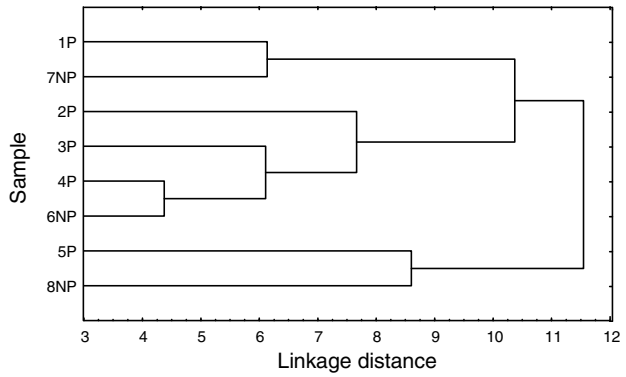

Fig. 4. Dendrogram from a hierarchical, single-link cluster analysis, showing the clustering of individual gas chromatographic profiles (in euclidian distances). Each sample is designated by a number and the symbol P for feather-pecked birds, and NP for non-feather-pecked birds.

than other chickens. Therefore, we cannot say with certainty that the olfactory differences between individuals are actually used by chickens in their social communication. However, other studies support this interpretation. For example, a recent study of sexual behavior in domestic chicken suggests that male mate choice involves olfaction and that the female's uropygial gland acts as a social odor cue (Hirao et al., 2009). Furthermore, other species of bird use olfactory information in various contexts (reviewed by Balthazart and Taziaux, 2009), particularly social recognition (Bonadonna et al., 2007; Bonadonna and Nevitt, 2004; Bonadonna et al., 2004; Hagelin et al., 2003). Uropygial gland secretions seem to be related to social and reproductive behavior in several species as studies have shown that the chemical composition of the secretions changes in the reproductive season (Bohnet et al., 1991; Piersma et al., 1999; Reneerkens et al., 2002; Soini et al., 2007). Composition is influenced by sex (Jacob et al., 1979), and in chickens it has been found that feather-pecking status, age and diet may all be related to the chemical profile of the secretion (Sandilands et al., 2004). Incidentally, our results also, as far as we are aware, provide the first demonstration of the ability of a mammal to discriminate between the odors of individual non-mammals.

The GC–MS analysis of our uropygial gland secretion samples verified the individual differences between samples detected by the mice (see Fig. 1). Every red junglefowl female showed a consistent and unique relative abundance of carboxylic acids, strengthening the suggestion of individual body odors among chickens (Hirao et al., 2009; Jones and Roper, 1997). Similar analyses of uropygial gland secretions have been done in LSL and ISA Brown chickens (Sandilands et al., 2004), juncos (*Junco hyemalis*) (Soini et al., 2007) and Antarctic prions (Bonadonna et al., 2007). The carboxylic acid profile of red junglefowl is similar to that for LSL and ISA Brown chicken reported previously (Sandilands et al., 2004) showing carboxylic acids ranging from C_{10} to C_{20}, with the highest percentage of C_{18} (see Fig. 3). In juncos, as in our red junglefowl females, there is a large interindividual variation in the relative abundance of carboxylic acids. Interestingly, in this species there is also some seasonal variation where the abundance of C_{12}, C_{14} and C_{16} differs between breeding and non-breeding conditions (Soini et al., 2007). Corresponding analyses of uropygial secretions have been performed in Antarctic prions showing that the chemical profile of a single bird is consistent from year to year, but different from that of another bird (Bonadonna et al., 2007), suggesting individual olfactory signatures in Antarctic prions, similar to our results.

The lack of systematic differences between pecked and non-pecked birds shown by both the discrimination abilities of the mice and the GC–MS analysis (see Figs 2 and 4) was unexpected, given the findings of Sandilands and colleagues (Sandilands et al., 2004). In that study, the proportions of C_{12}, C_{13} and C_{14} were significantly higher in P than in NP birds and C_{20} was higher in NP than in P birds. One possible reason for the discrepancy in the results might be that Sandilands and colleagues used a different breed of chicken (Sandilands et al., 2004). Perhaps domestication and selection have modified central aspects of the composition of gland secretion in relation to environmental stress. Another possible explanation for the discrepancy between the two studies might be that the housing conditions of the birds differed markedly with regard to floor substrate and cage size. It is of course still possible that chickens use individual odors for discriminating individual victims of feather pecking, but in red junglefowl we found no indications that potential victims as a group would have a common olfactory profile. We also cannot exclude the possibility that the birds may have had a different olfactory profile prior to the onset of feather-pecking, given we obtained samples from birds which had already been pecked for some time.

The next logical step for future studies of individual body odors in chicken would be to develop an appropriate behavioral assay to test: (1) whether chicken themselves are able to discriminate between conspecific body odors; (2) whether they indeed make use of this ability; and (3) if yes, in which behavioral contexts this may be of importance.

To conclude, trained mice were able to discriminate between odors of individual uropygial gland secretions from red junglefowl females. This indicates that red junglefowl have individual body odors, which was verified by GC–MS analysis showing interindividual differences in the relative abundance of carboxylic acids. However, we did not find any indication of a common signature among birds exposed to feather pecking.

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