Chemical fingerprints encode mother–offspring similarity, colony membership, relatedness, and genetic quality in fur seals

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Chemical communication underpins virtually all aspects of vertebrate social life, yet remains poorly understood because of its highly complex mechanistic basis. We therefore used chemical fingerprinting of skin swabs and genetic analysis to explore the chemical cues that may underlie mother–offspring recognition in colonially breeding Antarctic fur seals. By sampling mother–offspring pairs from two different colonies, using a variety of statistical approaches and genotyping a large panel of microsatellite loci, we show that colony membership, mother–offspring similarity, heterozygosity, and genetic relatedness are all chemically encoded. Moreover, chemical similarity between mothers and offspring reflects a combination of genetic and environmental influences, the former partly encoded by substances resembling known pheromones. Our findings reveal the diversity of information contained within chemical fingerprints and have implications for understanding mother–offspring communication, kin recognition, and mate choice.

chemical communication | mother–offspring recognition | GC-MS | genotype | pinniped

The chemical senses are the evolutionarily oldest and arguably most widespread means of interacting with the outside world. Olfaction in particular is fundamental to animal communication, mediating social interactions as varied as territorial behavior, kin recognition, and mate choice (1). Metabolomic tools, such as gas chromatography–mass spectrometry (GC-MS) have made it possible to generate individual-specific chemical "fingerprints." By separating compounds and quantifying their relative abundances, these fingerprints provide a wealth of information, even though not all compounds can necessarily be identified. Both volatile and contact cues are potentially hidden within the extreme complexity of chemical profiles, which is why a mechanistic understanding of chemical communication is still lacking in natural vertebrate populations (2).

In particular, "surprisingly little progress" has been made in understanding the link between vertebrate chemical fingerprints and genotype (2). Experimental studies have shown that females of several species are capable of discriminating potential partners based on olfactory cues (3–5). However, very few studies have demonstrated a convincing link between the molecular composition of chemical fingerprints and genetic traits, such as heterozygosity (a measure of genetic quality) and relatedness (6–9). These studies were almost exclusively conducted on a captive population of lemurs, a species known for its conspicuous use of scent marking.

A functional understanding of how genotype is chemically encoded also requires knowledge of how many and which types of substances are involved. This is challenging because, especially in natural populations, an individual's mixture of surface chemicals is not only the product of its genotype but may also be mediated by hormones, the microbial flora, body condition, and environmental factors (2). Thus, analyses based on overall chemical fingerprints may overlook

subtle genetic signatures and make little if any headway toward identifying the specific substances involved. A second less-appreciated problem is that the modest panels of around 10–15 microsatellite loci typical of most studies may be underpowered to detect genetic associations because they provide relatively imprecise estimates of both heterozygosity and relatedness (10, 11).

In arguably the only study to report a convincing link between chemical fingerprints and genotype in a natural vertebrate population, Leclaire et al. (9) used principle component analysis (PCA) to reduce chemical complexity. The authors identified a principle component in kittiwakes that correlated significantly with heterozygosity in both sexes and another that correlated with relatedness, but only in adult males. However, PCA iteratively maximizes the explained variance per component instead of seeking to capture the underlying structure and dimensionality of the data, which makes the resulting components hard to interpret (12). A better approach could be factor analysis (FA), a method from the field of psychology that estimates the latent variable structure of a dataset by dividing the total variability into that common to variables and a residual value unique to each variable (13). Statistical developments that allow FA to be applied to data with more variables than observations (14) have

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Significance

Understanding olfactory communication in natural vertebrate populations requires knowledge of how genes and the environment influence highly complex individual chemical fingerprints. To understand how relevant information is chemically encoded and may feed into mother–offspring recognition, we therefore generated chemical and genetic data for Antarctic fur seal mother–pup pairs. We show that pups are chemically highly similar to their mothers, reflecting a combination of genetic and environmental influences. We also reveal associations between chemical fingerprints and both genetic quality and relatedness, the former correlating positively with substance diversity and the latter encoded mainly by a small subset of substances. Dissecting apart chemical fingerprints to reveal subsets of potential biological relevance has broad implications for understanding vertebrate chemical communication.

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only recently made this approach amenable to studying chemical fingerprints.

Pinnipeds are an important group of marine mammals that provide an unusual opportunity to reveal insights into the basis of chemical communication. Studies of Steller's sea lions and harbor seals have revealed a large repertoire of functional olfactory receptor genes (15) and remarkably high olfactory sensitivity (16), respectively. Individuals of many pinniped species also have a strong musky smell that has been attributed to secretions of facial sebaceous and apocrine glands (17). These glands are known to hypertrophy during the mating season in at least two species (18), suggesting that olfactory cues may be particularly important during the reproductive phase of the life cycle.

Females of many otariid species breed in dense colonies and alternate lactation ashore with foraging trips at sea, necessitating accurate mechanisms for offspring localization and recognition (19). Although otariids use a combination of geographical, visual, auditory, and olfactory cues to find and recognize their pups (19), olfactory recognition is particularly important because females of many species accept or reject pups based on naso-nasal inspection (20, 21). Furthermore, a recent experiment on Australian sea lions (22) suggests that female pinnipeds are capable of discriminating filial from nonfilial pups using olfaction in the absence of other cues.

Antarctic fur seals (*Arctocephalus gazella*) provide a highly tractable model system for studying the importance of chemical cues in a free-ranging marine mammal. On Bird Island, South Georgia (Southwest Atlantic), a colony of fur seals has been studied intensively for over two decades (23). In this species, olfaction is known to be important for the close-range recognition of pups (20). However, females also show active mate choice for males who are both heterozygous and unrelated to themselves (24), raising the possibility that chemical cues might be involved not only in mother–offspring recognition, but also in mate choice.

Here, we combined GC-MS fingerprinting of skin swabs and genetic analysis to explore the chemical basis by which Antarctic fur seal mothers may recognize their pups. Because females of this species appear capable of choosing males based on heterozygosity and relatedness, we hypothesized that genotype should be chemically encoded and that this could provide a mechanism by which females could identify their pups. We therefore sampled mother–offspring pairs from two discrete but genetically indistinguishable colonies (see "colony differences" in *Results*), which in principle allows genetically encoded substances to be disentangled from those influenced by environmental differences between colonies. We also deployed over 40 microsatellite loci to enhance the power to detect associations between chemical fingerprints and genotype. Finally, we used FA together with a variety of nonparametric approaches to explore the structure of the chemical data and to uncover specific subsets of compounds associated with chemical differences between the colonies, mother–offspring similarity, and genetic relatedness.

Results

Chemical and Genetic Data. Chemical fingerprints and multilocus microsatellite genotypes were obtained for 41 mother–offspring pairs from two breeding colonies at Bird Island, South Georgia (Fig. 1). After removing compounds present in the control sample or only in a single individual, the total number of substances in each individual's chemical fingerprint averaged 35.9 and did not differ significantly between mothers and offspring (paired t test, $t = -0.05$, $P = 0.96$). All of the animals were genotyped at 43 highly polymorphic microsatellite loci, 41 of which did not deviate significantly from Hardy–Weinberg equilibrium (HWE) in either mothers or offspring after table-wide false-discovery rate (FDR) correction, and were therefore retained for subsequent analyses (Table S1). The mother-offspring pairs all had match probabilities of 100% (Table S2).
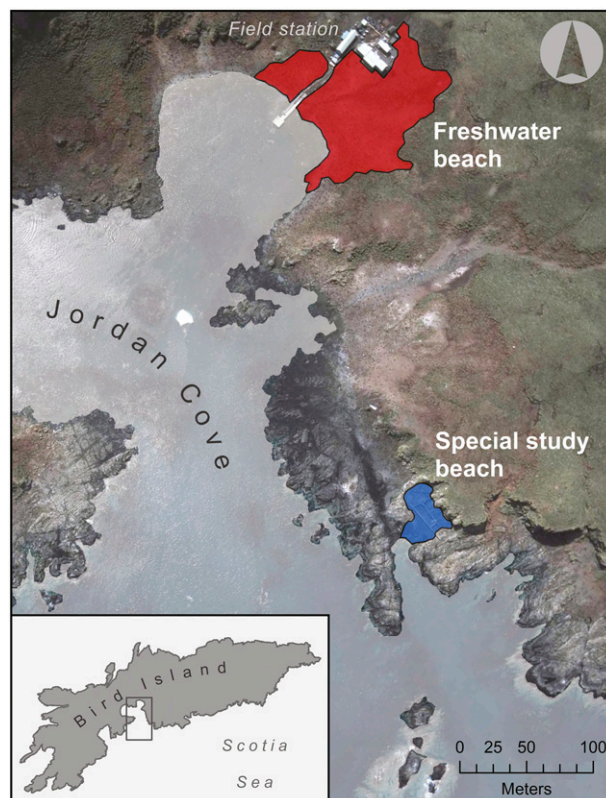

Fig. 1. Map of the study area showing the two breeding colonies from which Antarctic fur seal mother–offspring pairs were sampled. The red and blue areas demarcate freshwater beach and the special study beach, respectively.

Colony Differences. Multivariate statistical analysis of the relative proportions of each substance revealed highly significant differences between animals sampled from the two colonies, both overall (Fig. 2A) [analyses of similarities (ANOSIM), global $R = 0.57$, $P < 0.0001$] and separately for mothers (ANOSIM, global $R = 0.58$, $P < 0.0001$) and offspring (ANOSIM, global $R = 0.56$, $P < 0.0001$). Bayesian structure analyses of the genetic data yielded the highest average log-likelihood value for $K = 1$ in both mothers and pups (Fig. S1), indicating a lack of population structure. By implication, chemical differences between the colonies appear to reflect environmental influences (see *Discussion*).

Mother–Offspring Similarity. Pups were significantly more similar to their mothers in their chemical fingerprints than expected by chance (Fig. 2B), both overall (ANOSIM, global $R = 0.67$, $P < 0.0001$) and within each of the colonies (special study beach: ANOSIM, global $R = 0.53$, $P < 0.0001$; freshwater beach: ANOSIM, global $R = 0.45$, $P < 0.0001$). Chemical similarities between mothers and offspring could be encoded by shared genes or might simply reflect their spatial proximity. However, we found no relationship between chemical similarity and geographic distance within the special study beach, where pupping locations are recorded to the nearest square meter, either for mothers (Mantel's $r = 0.008$, $n = 20$, $P = 0.44$) or offspring (Mantel's $r = 0.06$, $n = 20$, $P = 0.31$). This finding suggests that chemical similarity is not associated with geographic proximity per se.

Genotype and Overall Chemical Fingerprints. To determine whether genetic relatedness is reflected in chemical similarity, we tested for an association between pairwise r (see Table S3 for summary statistics) and Bray–Curtis similarity. A highly significant
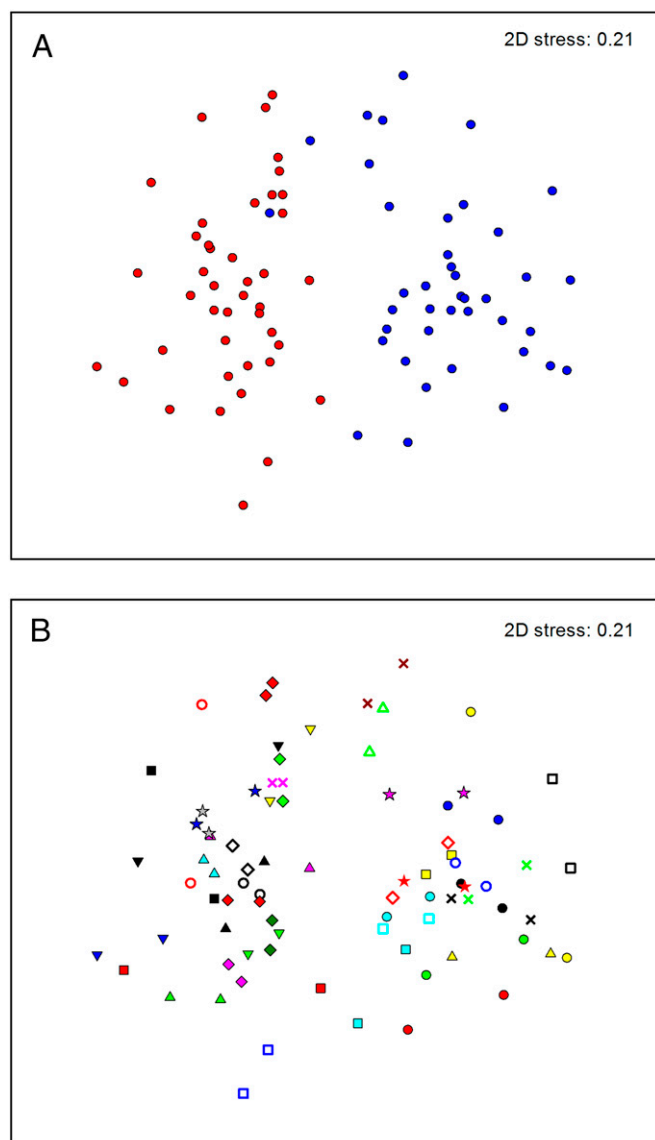
Fig. 2. Two-dimensional nonmetric multidimensional scaling plots of chemical fingerprints of 41 Antarctic fur seal mother–offspring pairs. Bray–Curtis similarity values were calculated from standardized and log(x+1)-transformed abundance data; (*A*) color-coded by colony (red points: freshwater beach; blue points: special study beach); (*B*) plotted by mother–offspring pair, with each pair being denoted by a different symbol/color combination. The scales of the two axes are arbitrary. The closer the symbols appear on the plot, the more similar the two chemical fingerprints are.

relationship was obtained when all of the animals were analyzed together (Mantel's $r = 0.07$ $n = 82$, $P = 0.005$) but nonindependence of both chemical and genetic data for mothers and offspring may introduce pseudoreplication. We therefore repeated the analysis separately for mothers and offspring, finding no significant relationships (mothers, Mantel's $r = 0.06$ $n = 41$, $P = 0.10$; offspring, Mantel's $r = 0.030$ $n = 41$, $P = 0.25$).

To test for a chemical signal of genetic quality, we regressed the number of compounds in an individual's chemical fingerprint, a measure of chemical complexity, on standardized multilocus heterozygosity (sMLH). A significant positive correlation was found in mothers (Fig. 3) ($F_{1,40} = 5.26$, $P = 0.026$) but not in offspring ($F_{1,40} = 0.50$, $P = 0.483$). The strength of correlation also increased steadily with the number of microsatellites deployed in mothers and to a lesser extent in offspring (Fig. 4*A*).

Conversely, the estimation error of the parameter g_2, which quantifies the extent to which heterozygosities are correlated across loci, decreased with increasing marker number (Fig. S2). Overall, g_2 was significantly positive (0.0022, $P = 0.032$ based on 1,000 iterations of the dataset), indicating that heterozygosity is correlated across the genome.

Factor Analysis. Chemical fingerprints are highly complex and may contain numerous compounds influenced by nongenetic factors. We therefore used principal axis FA to decompose the multidimensional chemical data into four factors (see *Methods* for details). Fitting the scores of all four factors together in a generalized linear model (GLM) of maternal heterozygosity, factors 1 and 2 were retained as significant predictor variables (Table 1, mother's sMLH) and together explained almost twice as much deviance as the number of compounds in an individual's chemical fingerprint (23.4% vs. 11.9%, respectively). A simple GLM of sMLH fitting the sum of the two factors as a single explanatory variable explained roughly the same amount of deviance (23.4%, $F_{1,39} = 11.91$, $P = 0.001$). In contrast, none of the factors were significantly associated with offspring heterozygosity.

To test whether any of the factors are also associated with genetic relatedness, we used partial Mantel tests to derive the statistical significance of each factor while controlling for the others (see *Methods* for details). Factor 1 was significantly correlated with relatedness in mothers (Mantel's $r = -0.123$, $n = 41$, $P = 0.028$) (Fig. S3) but not in offspring (Mantel's $r = 0.024$, $n = 41$, $P = 0.65$). None of the other factors correlated significantly with relatedness in either mothers or offspring. As with the signal of heterozygosity, the strength of association between factor 1 and relatedness increased steadily with marker number (Fig. 4*B*).

We next constructed a GLM to test for differences in the values of each of the four factors between the two colonies (Table 1, colony). Factors 1, 2, and 3 did not differ significantly, whereas factor 4 exhibited a highly significant difference between the colonies (Fig. 5). Thus, factors 1 and 2 both show correlations with genetic traits as well as overlapping distributions between colonies, factor 3 is not significantly associated with any of the variables we measured, and factor 4 represents substances that discriminate the two colonies and must therefore be environmentally influenced.

Identification of Important Substances. To identify substances that contribute most strongly toward chemical similarity within mother–offspring pairs, we used the "similarity percentages" routine (SIMPER, see *Methods*). Selecting the two most important
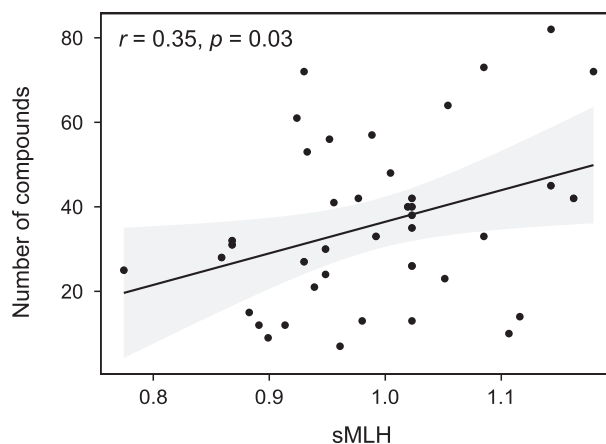
Fig. 3. Relationship in mothers between sMLH and the number of compounds in an individual's chemical fingerprint.
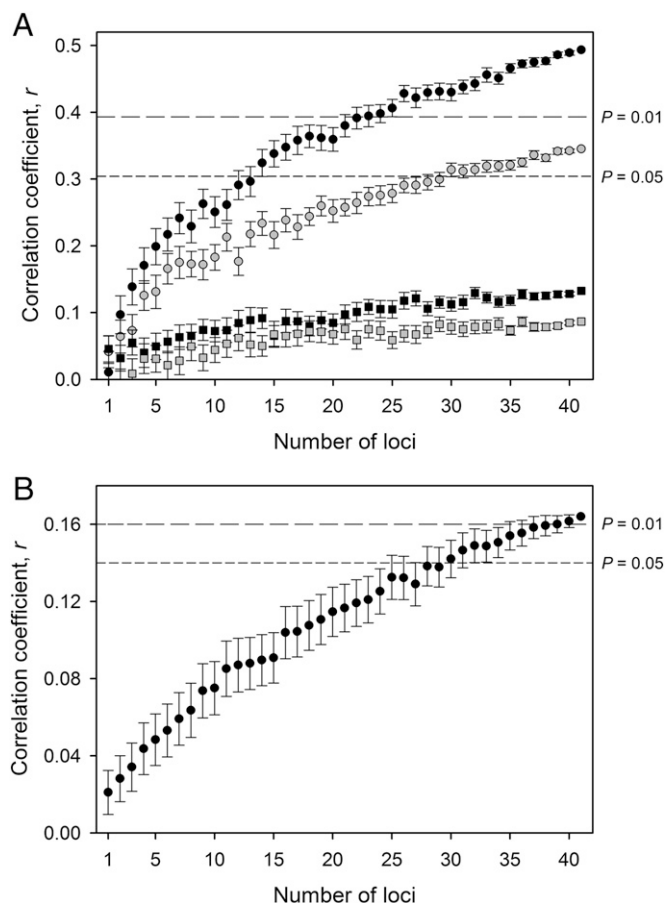
Fig. 4. Dependency of the strength of genetic associations on the number of randomly sampled microsatellite loci. Strength of association was quantified as the correlation coefficient (r) between (*A*) sMLH and the number of compounds in an individual's chemical fingerprint (gray symbols) and the sum of an individual's factor 1 and factor 2 values (black symbols), plotted separately for mothers (circles) and offspring (squares); (*B*) relatedness and Bray–Curtis similarity at the 10 best substances in mothers (see *Methods* for details). Mean ± SE of five resamplings of the data are shown for each point. The dashed lines represent significance thresholds.

compounds for each of the 41 mother–offspring pairs, we identified a total of 12 substances (Table S4, mother–offspring similarity). These substances yield a much stronger pattern of within-pair mother–offspring similarity (ANOSIM, global $R = 0.68$, $P < 0.0001$) than was obtained for the full dataset. Similarly strong patterns were obtained separately for each of the colonies (special study beach: ANOSIM, global $R = 0.53$, $P < 0.0001$; freshwater beach: ANOSIM, global $R = 0.31$, $P = 0.001$).

We also used SIMPER to search for substances accounting for most of the chemical dissimilarity between the two colonies. This approach identified a total of 15 substances (Table S4, colony dissimilarity) that collectively yield a much higher global R value (ANOSIM, global $R = 0.77$, $P < 0.0001$) than was obtained for all of the chemicals. To identify substances associated with genetic relatedness, we used the BIO-ENV procedure embedded in a bootstrap framework (see *Methods* for details). We obtained a subset of 10 substances (Table S4, relatedness) that consistently occurred within the "best" subsets (i.e., maximizing the relationship between chemical similarity and relatedness) over all 10×10^6 bootstrap samples and collectively maximized the relationship between chemical distance and relatedness (Fig. S4). Chemical similarity based on these 10 substances was signifi-

cantly associated with genetic relatedness (Mantel's $r = 0.164$, $n = 41$, $P = 0.001$).

Finally, we cross-referenced the three lists of substances to evaluate any potential overlap. Of the 12 compounds carrying the strongest signal of mother–offspring similarity, 9 also occurred in the subset of chemicals that differ between the two colonies, implying that they may be influenced by environmental conditions ashore. Remarkably, an additional two compounds overlapped with the best subset of chemicals associated with genetic relatedness. The mass spectra and Kovats indices of these substances indicate close resemblance to the known pheromones ethyl-9-hexadecenoate and heptadecanoic acid (see *Discussion* and Table S4, relatedness).

Discussion

Although mother–offspring recognition is under strong selection in many species, little is known about its chemical basis, particularly in natural populations of nonmodel organisms. We show that fur seal pups are highly similar to their mothers in their chemical fingerprints and that this similarity is largely encoded by a handful of substances that also carry information about either colony or genotype. Our findings provide intriguing insights into how females could use chemical information to recognize their offspring and may also help to explain how fur seals appear capable of exercising mate choice for heterozygous and unrelated partners (24).

Our study was partly motivated by the discovery that female Australian sea lions can identify their pups using only olfactory cues (22). In most vertebrate species, chemical fingerprints show marked differences by sex, age, and reproductive status (25), a pattern that is partly reflected in our data because it is only the mother's chemical fingerprints that encode genotype. However, the overall chemical fingerprints of mothers and offspring are still very similar, raising the possibility that self-referent phenotype matching (26) could be used in mother–pup recognition. This is a conceptually simple mechanism by which the own phenotype is a representation or template used for the recognition of relatives. Self-referent phenotype matching has been demonstrated in a variety of mammalian, bird, and fish species (27). However, further experimental evidence would be needed to show that mother–offspring recognition in fur seals relies on self-matching rather than social learning. Interestingly, allosuckling rates vary considerably among pinniped species, from 6% in New Zealand sea lions to up to 90% in Hawaiian monk seals, suggesting that mother–pup recognition abilities may vary among species (28). The Antarctic fur seal has one of the lowest observed rates of allosuckling (29), which is consistent with the strong pattern of chemical similarity we find between mothers and their pups.

Although chemical fingerprints are widely assumed to encode genetic traits, such as relatedness and individual heterozygosity,

Table 1. Generalized linear models of sMLH in mothers and the colony from which an animal was sampled

Term	Slope	F	df	P
Mother's sMLH ($n = 41$, total explained deviance = 23.40%)				
Factor 1	0.028	5.69	1	0.022
Factor 2	0.028	5.83	1	0.021
Colony ($n = 82$, total explained deviance = 56, 26%)				
Factor 4	0.38	102.88	1	<0.0001

All four factors were initially fitted together as explanatory variables and the models were then reduced by sequentially removing the least-significant term until only significant terms were retained (see *Methods* for details). The F values for each term represent the change in deviance after removing that term from the model.
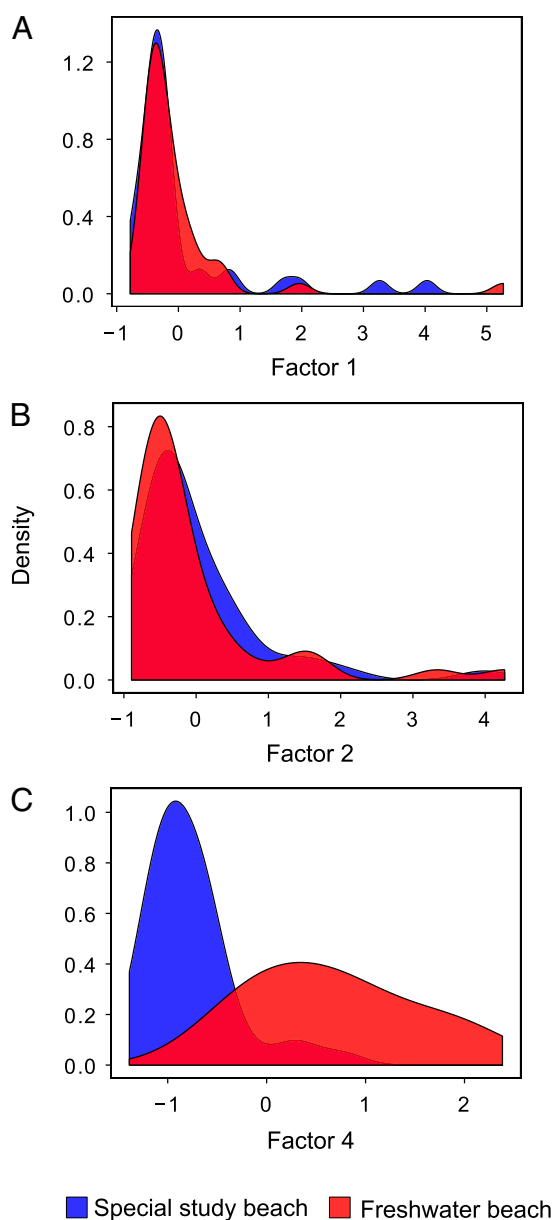
Fig. 5. Distribution of factor scores of individuals sampled from the two seal colonies. Factors 1, 2, and 4 are shown in *A*, *B*, and *C*, respectively. Freshwater beach is shown in red and the special study beach is shown in blue.

only a handful of studies have reported the expected associations. Moreover, chemical profiles typically change with age and reproductive status (25) and genetic correlations have, to our knowledge, only been detected in breeding adults (6, 7). Analyzing the relationship between heterozygosity and chemical complexity separately for mothers and pups shows a clear correlation that increases with the number of loci for mothers, a pattern that is weak or lacking in pups (Fig. 4*A*). Because of the consistency of our results with the literature, we believe this reflects a genuine functional difference between the chemical fingerprints of mothers and pups.

We also find a marked difference in the way that heterozygosity and relatedness are encoded in chemical fingerprints. Heterozygosity is detectable in the overall fingerprint, as it is correlated with the number of chemicals, whereas relatedness is encoded by a small subset of chemicals, whose signal is diluted by analyzing the overall chemical fingerprint. The diversity of chem-

icals reflected in heterozygosity could be the result of genetic polymorphisms in the enzymes involved in the synthesis of semiochemicals (6) but may also be influenced by condition dependent factors (see below). In contrast, it makes sense that genetic relatedness could be encoded by a small subset of chemicals that potentially reflect certain genes, such as the MHC, a highly polymorphic cluster of immune genes detectable through scent (30, 31).

In natural populations, environmental effects on chemical fingerprints are likely to be particularly strong. The only study of a free-ranging, natural population to have detected an association with genotype used PCA to reduce the dimensionality of the chemical data (9). However, this approach is not ideally suited to detecting such signals because a principal component that explains maximal variance may not necessarily provide an optimal representation of the underlying genotype. We applied PCA to our dataset but obtained no significant correlations between any of the resulting principal components and relatedness, and a weaker signal of heterozygosity than was obtained using FA. This result could be because of the so called "simple structure" that is obtained by rotation of the factors within FA (32). This results in each substance loading primarily on a single factor and not on the others, meaning that the factors represent subsets of variables that covary and are therefore likely to have a shared basis, such as genes or the environment.

FA was considerably more successful than PCA at detecting patterns relating to genotype within our chemical dataset. Factors 1 and 2 together explained almost twice as much of the deviance in heterozygosity as a simple regression on the number of substances, and relatedness was significantly associated with factor 1 but not with Bray–Curtis similarity based on the overall fingerprints. Because each factor mostly represents a subset of the total pool of chemicals, this finding is consistent with Hurst and Beynon's suggestion that the selective assessment of specific semiochemicals may allow individuals to assess genotype more accurately than from entire chemical fingerprints (2).

It is unclear why factor 1 carries information about both heterozygosity and relatedness, whereas factor 2 correlates only with heterozygosity. One possibility is that heterozygosity and relatedness are to some extent signaled by the same substances, potentially deriving from the MHC. As the substances loading on factor 2 are essentially uncorrelated with those loading on factor 1, we speculate that heterozygosity may influence the chemical fingerprint through two or more different pathways. Factor 1 could thus represent a direct pathway from genes to the chemical fingerprint, whereas factor 2 may represent an indirect pathway where body condition or the microbiome could be possible mediators. Future work will aim to explore these possibilities.

An important strength of our study was a sampling design that facilitated disentangling genetically encoded substances from those influenced by the environment. We found that factors 1 and 2, which both encode some aspect of genotype, did not differ significantly in the distribution of factor scores between the colonies, whereas factor 4, which carried no discernible genetic information, showed a highly significant difference. These differences could either be a result of environmental chemicals that directly contribute toward the profile, or could reflect alterations to the chemical fingerprint caused by different conditions on the beaches (e.g., temperature, wind, solar radiation). We would need to sample more colonies to determine the concrete causes.

Another important aspect of our study design was the unusually high genetic resolution provided by 41 microsatellites. Most studies use around 10–15 loci, which for our dataset was insufficient to detect a significant correlation between maternal heterozygosity and compound richness (Fig. 4*A*). However, the strength of correlation increased steadily as more microsatellites were deployed until a highly significant relationship was obtained with the full marker panel. Similarly, the error with which the parameter g_2 was estimated from the genetic data decreased

steadily with increasing marker number. This finding is consistent with the suggestion that, as long as heterozygosity is correlated across the genome (as is the case where appreciably inbred individuals are present), increasing the number of markers should improve the estimation accuracy of genome-wide heterozygosity, leading to a strengthening of effect size (10, 33). A similar pattern was also obtained for genetic relatedness, suggesting that, if many thousands of genetic markers could be deployed, an even greater proportion of the chemical variance should be explicable by genotype (11).

In many species, heterozygosity is associated with fitness (34). In Antarctic fur seals, multilocus heterozygosity at nine microsatellites correlates with early survivorship and breeding success in females (35), as well as reproductive success in males (36). Females of this species also appear to exert mate choice based on their partner's genotype (24) but it is unclear how this could be achieved. The finding that heterozygosity and relatedness are both encoded in mother's chemical fingerprints lends support to the hypothesis that chemical cues could be involved, although unfortunately we were not able to include adult males in this study because they are challenging to capture and sedate. Nevertheless, as male fur seals emit a strong musky odor (17), which has been proposed to attract females during the mating season (37), it seems plausible that genotype could also be encoded in adult male chemical fingerprints.

To explore the extent to which genes and the environment influence mother–offspring similarity, we first attempted to identify the most important substances associated with mother–offspring similarity, colony dissimilarity, and genetic relatedness. We obtained relatively small subsets of 12, 15, and 10 chemicals, respectively. In the case of mother–offspring similarity and relatedness, these subsets yielded much stronger associations than were obtained for the overall fingerprints. This result suggests that SIMPER and BIO-ENV were successful in identifying important chemicals within the total set of 213 substances, although this does not preclude additional chemicals playing a lesser role. It is also noteworthy that as many as 10 or more chemicals appear to encode relatedness, given that a single locus is expected to provide little power to distinguish anything other than close relatives (2).

Evaluating the overlap between the subsets of chemicals associated with mother–offspring similarity, colony dissimilarity, and genetic relatedness revealed an interesting pattern. Of the top 12 substances accounting for the similarity between mothers and their pups, 9 also occurred in the subset of chemicals that showed the greatest differences between the two colonies. Although our analysis is not exhaustive, as we focused only on the most important substances, this nevertheless suggests that chemical similarity within mother–offspring pairs is strongly influenced by the local environment. A further two substances also overlapped with the subset of chemicals associated with genetic relatedness, implying that mother–offspring similarity also has a genetic basis. Both of these substances reveal similarity to known pheromones, consistent with the previous suggestion that pheromone-like chemical signals may play an important role in mother–offspring recognition across a variety of taxa (38).

Little is currently known about the specific chemicals that signal genetic relatedness in vertebrates (2). Although we were only able to putatively identify three of the top 10 substances encoding relatedness using the National Institute of Standards and Technology (NIST) database, the mass spectra and Kovats indices of these compounds reveal close resemblance to the known pheromones ethyl 9-hexadecenoate, heptadecanoic acid and ethyl stearate (Table S4, relatedness). According to the pherobase database, all three of these substances are part of the chemical communication system of a variety of different taxa, ranging from bumblebees to badgers. Heptadecanoic acid, for example, is a known pheromone of 33 different species, including 26 vertebrate taxa. However, to act as a pheromone in a given species, a

chemical must meet a number of strict criteria (39), which would require experimental evidence (see below).

Although we captured a large number of substances of varying volatility, we only recovered compounds soluble in ethanol and which could be detected by GC-MS. Extraction with other solvents was not possible because of logistic reasons. Nevertheless, even though our sampling of chemicals is likely to be incomplete, our analyses revealed a number of statistically significant and potentially biologically relevant patterns. In addition, we detected a number of chemicals that may carry important information. However, because some of these substances may have been further metabolized after extracting them from the skin (40), we cannot exclude the possibility that some of the putatively identified compounds could be breakdown products.

Finally, biologically relevant chemical cues can be transferred in a variety of ways, from volatile substances recognized by olfaction to chemicals that act when two individuals are in physical contact (41). Because adult female fur seals and their pups conduct naso-nasal inspections during the recognition procedure (20), it is possible that some of the chemicals may act through contact. To unequivocally determine the biological relevance of the chemicals we have identified, as well as their precise mode of action, would require behavioral assays in the field. This will be challenging, but our results provide the basis for testable hypotheses on potential chemical signals and the substances involved.

Methods

Study Site and Field Methods. Forty-four mother–offspring pairs were sampled from two breeding colonies: freshwater beach and special study beach, separated by ∼200 m (Fig. 1) on Bird Island, South Georgia (54° 00′ S, 38° 02′ W). Breeding females and their pups were captured and restrained on land using standard methodology (42). Seal capture and restraint were part of annual routine procedures of the Long Term Monitoring and Survey program of the British Antarctic Survey. We obtained chemical samples by rubbing the cheek, underneath the eye, and behind the snout with a sterile cotton wool swab. Each swab was individually preserved in a glass vial in 60% (vol/vol) ethanol stored at −20 °C. All of the samples were obtained immediately after capture by the same team of two seal scientists at both colonies. Tissue samples for genetic analysis were collected as described by Hoffman et al. (43) and stored individually at −20 °C in the preservative buffer 20% (vol/vol) DMSO saturated with salt. Fieldwork was approved by the British Antarctic Survey Ethics Review Committee. Samples were collected and retained under permits issued by the Department for Environment, Food and Rural Affairs (DEFRA), and in accordance with the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

Chemical Analyses. We first took 1 mL of each sample and allowed the ethanol to evaporate at room temperature under a fume hood for a maximum of 12 h before resuspending in 50 µL dichlormethane for subsequent processing. The samples were then analyzed on a GC equipped with a VF-5 ms capillary column (30 m size × 0.25 mm inner diameter, DF 0.25, 10-m guard column; Varian) and coupled to a quadrupole mass spectrometer (Focus GC-DSQ MS system, Thermo Electron). A blank sample (control with cotton wool and ethanol) and an alkane mix (C8–C28) were analyzed as well. One microliter of each sample was injected into a deactivated glass wool-packed liner at an inlet temperature of 225 °C and processed in a splitless mode. Carrier gas (He) flow rate was held at 1.2 mL/min. The GC run was initiated at 60 °C for 3 min then ramped at 10 °C/min to 280 °C, where it remained for 20 min. The transfer line temperature was set to 280 °C and mass spectra were taken in electron ionization mode at 70 eV with five scans per second in full-scan mode (50–500 m/z). GC-MS data were processed using the program Xcalibur (Thermo Scientific). To ensure that the scoring of compounds was as objective as possible, we wrote a custom R script (available on request) that compensated for minor shifts in retention times among chromatograms by maximizing the number of shared components between samples through very small (≤0.03 ms) shifts in the retention time. To double-check the reliability of the scoring, ∼10% of compounds were selected at random and scored by eye.

Genetic Analysis. Total genomic DNA was extracted from each sample using a standard phenol-chloroform protocol and genotyped at 43 highly polymorphic microsatellite loci (see Table S1 for details). These were PCR-amplified in eight separate multiplexed reactions using a Type It Kit (Qiagen) as

described in Table S1. The following PCR profile was used: one cycle of 5 min at 94 °C; 24 cycles of 30 s at 94 °C, 90 s at T_a °C and 30 s at 72 °C; and one final cycle of 15 min at 72 °C (see Table S1 for T_a). Fluorescently labeled PCR products were then resolved by electrophoresis on an ABI 3730xl capillary sequencer and allele sizes were scored automatically using GeneMarker v1.95. To ensure high genotype quality, all traces were manually inspected and any incorrect calls were adjusted accordingly.

Genepop (44) was used to calculate observed and expected heterozygosities and to test for deviations from HWE, separately for mothers and pups, specifying 10,000 dememorizations, 1,000 batches, and 10,000 iterations per batch. Two loci that deviated from HWE in either mothers or pups after table-wide correction for the FDR using Q-value (45) were excluded from subsequent analyses, leaving a total of 41 loci (Table S1). Because milk stealing is common in fur seals and can lead to errors in the assignment of mother–offspring pairs in the field (29), we used the program Colony v2.0.5.0 (46) to verify that all of our mother–offspring pairs were genuine. Coancestry v1.0.1.2 (47) was then used to generate a pairwise relatedness matrix based on Queller and Goodnight's statistic, r (48). Each individual's heterozygosity was expressed as sMLH, which is defined as the total number of heterozygous loci in an individual divided by the sum of average observed heterozygosities in the population over the subset of loci successfully typed in the focal individual (49). The two-locus heterozygosity disequilibrium g_2, which measures the extent to which heterozygosities are correlated across loci, was then computed using the method of David et al. (50). Sensitivity of this estimate to the number of loci was explored by randomly selecting different sized subsets of loci and recalculating g_2 1,000 times.

To test for population structure, Bayesian cluster analysis of the microsatellite dataset was implemented using Structure v2.3.3 (51). Structure uses a maximum-likelihood approach to determine the most likely number of genetically distinct clusters (K) by subdividing the dataset in a way that maximizes HWE and minimizes LD within the resulting clusters. Separately for mothers and pups, we ran five independent runs for each value of K ranging from 1 to 10 using 1×10^6 Markov chain Monte Carlo iterations after a burn-in of 1×10^5, specifying the correlated allele frequencies model and assuming admixture. The most likely K was then evaluated using the maximal average value of Ln $P(D)$, a model-choice criterion that estimates the posterior probability of the data.

Statistical Analysis Framework. Any chemicals appearing in the control sample or present in only one sample were excluded from further analyses, leaving a total of 213 substances. To explore the completeness of our sampling, we estimated the maximum number of substances present in the population using the Michaelis–Menten Function, based on a permutation procedure (9,999 iterations). Up to 229 substances might be expected in a larger sample of individuals, suggesting that we have sampled around 95% of all potential substances. Analyses were conducted on the relative proportion of each substance (%) to the total amount of substances (52). We then used a three-step analytical framework to: (*i*) visualize and statistically analyze overall patterns of chemical fingerprint similarity in relation to breeding colony, mother-offspring pair, relatedness, and heterozygosity; (*ii*) tease out subsets of chemicals containing genotypic and environmental information; and (*iii*) identify specific compounds involved. Computer code and documentation are provided as a PDF file written in Rmarkdown (Dataset S1) together with the data (Dataset S2).

Overall Patterns of Chemical Similarity. The chemical fingerprint data were visualized using nonmetric multidimensional scaling (53) based on a matrix of pairwise Bray–Curtis similarity values calculated from the log(x+1)-transformed data. This approach allows visualization of a high-dimensional chemical similarity space by placing each individual in a 2D scatterplot such that ranked between-individual distances are preserved, points close together representing individuals with relatively high chemical similarity. Differences between a priori defined groups (i.e., the breeding colonies and mother–offspring pairs) were then analyzed through nonparametric ANOSIM (53) using 99,999 iterations of the dataset. ANOSIM is a permutation test that provides a way to evaluate whether there is a significant difference between two or more groups of sampling units without the need for assumptions concerning data distribution or homoscedasticity. These analyses were implemented in R using the vegan package (54).

Factor Analysis. To dissect apart genetic from environmental components, we performed a principal axis FA on the chemical data. We used an oblique rotation technique (promax), which allows the factors to be correlated. This type of rotation was used because it is possible that certain compounds within the chemical fingerprint may encode more than one genetic characteristic (e.g., heterozygosity and relatedness) and could thus be correlated with more than one factor. FA cannot be applied when a dataset has more variables than observations (D>>N) because the covariance matrix is singular and an inverse cannot be computed. We therefore used the function factor.pa.ginv() from the R package HDMD, which uses a generalized inverse matrix (14). An important step in factor analysis is choosing a reasonable number of factors to represent the data (32). As our dataset is complex and contains many zero entries, some common methods like parallel analysis may lead to an impractically large number of factors. Consequently, we applied two methods for determining the optimal number of factors. First, we used the Bayesian Information Criterion, which optimizes the trade-off between model complexity and model fit, and second we used a scree plot, which visually depicts the drop in the factor eigenvalue course (32, 55). Both methods suggested four factors.

Generalized Linear Models. To explore the contributions of each of the four factors toward the signal of heterozygosity, we constructed separate GLMs of mother and offspring sMLH, in which we fitted all four factors together and specified a Gaussian error structure. We then tested for factors that differ significantly between the two colonies by constructing a GLM with colony as the response variable (modeled using a binomial error structure) and the values of the four factors fitted as predictors. For each GLM, we initially implemented a full model containing all of the predictor variables and then used standard deletion testing procedures based on F tests (56) to sequentially remove each term unless doing so significantly reduced the amount of deviance explained.

Partial Mantel Tests. To test for associations between each of the factors and genetic relatedness, we used the relatedness matrix based on all 41 loci as the response variable and fitted as predictor variables matrices of pairwise similarity at each of the four factors using a Partial Mantel test implemented in the ecodist package (57). This randomizes the rows and columns of one dissimilarity matrix but leaves the others unpermuted. Separate models were constructed for mothers and offspring, each using 10,000 permutations of the dataset. Finally, we computed the Spearman rank correlation (Mantel's r) and two-tailed P value for the association between relatedness and a factor matrix given the other factors as covariates.

Identification of Chemicals. We next attempted to identify specific chemicals associated with breeding colony, mother–offspring similarity, and genetic relatedness. First, we assessed the contributions of specific substances to the similarity within groups, using the "similarity percentages" routine (SIMPER) (58). This process decomposes all Bray–Curtis similarities within a group into percentage contributions from each compound, listing the compounds in decreasing order of importance. As groups, we specified (*i*) the two breeding colonies and (*ii*) the 41 different mother–offspring pairs.

Second, to explore the contributions of individual chemicals to the signal of genetic relatedness, a continuously distributed variable, we used the BIO-ENV procedure (58) to identify the "best" subset of compounds within the chemical abundance matrix that maximizes the rank correlation between pairwise Bray–Curtis similarities and relatedness. However, with over 200 different chemicals being present in the chemical data matrix, it seems likely that this approach could yield spurious associations, especially given that some of the chemicals were present only in a few individuals. For this reason, we embedded the BIO-ENV procedure in a bootstrap analysis as follows: (*i*) we randomly subsampled 20 of the 41 mothers 20,000 times; (*ii*) for each subsample, we randomly selected 10 chemicals, each 500 times; (*iii*) for each of the resulting 10×10^6 subsamples, comprising 20 individuals and 10 compounds, we applied the BIO-ENV procedure and saved the compounds present in the best subset. We then summed up the occurrences of every chemical throughout all of the subsets and sorted them in decreasing order to represent their relative importance. The basic assumption of our approach is that random correlations will not be consistent over the different subsamples of individuals and compounds, whereas compounds that genuinely encode relatedness should be recovered consistently across many subsets. This procedure was conducted in R using the bio.env() function in the sinkr package (59).

Identification of putative substances encoding mother-pup similarity, colony differences, and relatedness were based on two steps: (*i*) comparing the mass spectrum of a specific substance with the best match of the NIST library (NIST 2005 and 2008) and (*ii*) calculating the Kovats Retention Index and comparing this to the literature value (obtained from www.Pherobase.com and www.chemspider.com). Kovats Indices (60) were calculated by running a sample of linear alkanes (C8–C28) under the identical GC-MS conditions as described above.

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