Elephant Breath
Clues about health, disease, metabolism and social signals

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Introduction
Elephants have an unusual respiratory set-up for a terrestrial mammal, perhaps indicative of their postulated aquatic origin (West, 2001, 2002; Gaeth et al, 1999). These remarkable attributes involve a precocious fetal development of an eventually lengthy trunk through which 70-80% of breathing occurs, the prenatal fusion of parietal and visceral pleura resulting in the lack of a separate pleural cavity, and a tremendous lung surface area. We have exploited these unusual attributes to monitor gaseous compounds released in expired breaths in a non-invasive manner, searching for indications of health or disease, normal or abnormal metabolic events and chemical compounds that may serve as social signals.

The anatomical basis of the respiratory apparatus of elephants – the lungs, trachea and nasal passageways – has some specialized components. The long, up to 3 meters, truncal passageway effectively warms air well in advance of reaching the trachea; it also functions to cool the departing carbon-dioxide laddened expiring air. After transiting the trachea, which is about 30 cm in length and 5-7 cm in diameter (in Loxodonta africana; Sikes, 1977), the incoming, warmed air enters large, bilobed lungs. The edges of the lungs are more rounded and thus less multi-lobular than other mammals and the right lung, usually larger than the left, may have a secondary lobe. The lack of a well-defined pleural cavity is significantly different from other mammals and perhaps related to the suggested aquatic origin. The lungs are attached by masses of tough white connective tissue to the inside of the chest wall and to the diaphragm. It is the movements of the diaphragm that cause the lungs to expel or gather air (Todd, 1913); after passing through the trachea, incoming air enters progressively smaller ducts, termed alveolar ducts, to reach small sacs called alveoli (Engel, 1952).

In these lung alveoli, rapid, effective exchange of gases and volatile metabolites from the circulatory system to the outside take place via expired air. There is a broad, extensible surface area for gas exchange (Engel, 1963). The membrane separating the air in the lungs from the blood is thin enough to allow oxygen to diffuse readily into the blood, and carbon dioxide to move in the opposite direction. Each lung alveolus sits in a capillary network composed of large pulmonary capillaries with multiple anastomoses. The thin membranes – capillary and alveolar – are separated by small interstitial spaces and surround each lung alveolus. Only this thin, several cells thick, non-muscular endothelial

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barrier separates the lung air space from the circulatory system. In a human, the total area of these alveolar walls in contact with the capillaries is about 90-100 square meters; in an elephant it is guesstimated, based on lung weights (Benedict, 1936) and degree of complexity, to be 5000 square meters. In most mammals a cubic centimeter of lung tissue will stretch out to 800 cm² of surface area, so a human lung can be stretched to an area the size of a tennis court. As lung volume is constant in mammals at about 5% body weight (Gehr et al., 1981), the elephant lung would stretch to cover a soccer field. In addition the lung surface is also coated with surfactants that reduce surface tension (Clements et al., 1970) and may aid in transport across the thin membraneous layers. Such a system ensures the rapid transfer of carbon dioxide and small organic molecules from the internal blood system to the lung air media.

During breathing, much, but not all of the lung air is expelled. In humans a particular breath is about 500 ml in volume. The first third of the expelled air is a mixture of recently inspired and older expired air, and the latter ⅔ represents true “tidal” or alveolar air. In land mammals the respiratory and digestive systems share some orifices and passageways - thus in mouth breathing mammals, mouth components may contribute compounds to the expired air volatiles. Uniquely, in many marine mammals the pulmonary and digestive systems are completely separate, thus the expired air is only a reflection of metabolites from the blood crossing into the lungs, from the lungs themselves, and possible exudates from the epithelial linings of various passageways and may include bacterial products. The elephant respiratory system is a scenario approaching that of marine mammals. Mouth breathing in elephants is considered “supplemental” as most breathing occurs via the truncal passages directly from the lungs (Figure 1).

Methods
Using the unusual anatomical and physiological elephant functions, we have developed methodology:
1. to collect the exhalant breath of individual elephants, 2. to confirm its reflection of tidal air by the percentage of carbon dioxide (ambient air=0.033%, totally expired air=5% CO₂), 3. to identify more than 125 volatile organic components in breath. Such analyses allow the detection of volatiles of fun-
gal or bacterial origin along the respiratory tract, as well as the characterization and quantitation of metabolic products of blood origin that offer information on health or may be the source of social chemical signals, including those emitted during musth.

Thus the purposes of our breath analyses are threefold: first, such analyses have immediate health relevant information about possible fungal or bacterial conditions either along the air passageways or in the lungs themselves. Complimenting and supporting our studies on the health aspects of organic compounds in elephant breath are our comprehensive

studies of measuring volatile organic compounds in the exhalant breath of a wide variety of mammals under a broad spectrum of wild and captive conditions. Our published and unpublished studies of breath include the Asian *Elephas maximus* and African *Loxodonta africana* elephants, as well as studies on the elephant’s close relative, the manatee, *Trichechus manatus* (Figure 2), and a variety of cetaceans that are particularly susceptible to respiratory infections: the bottlenose dolphin, *Tursiops truncates*, several species of sperm whales, the killer whale, *Orcinus orca*, and a number of terrestrial ungulates. Our expertise is reflected in state-of-the-art analytical systems in our laboratory capable of identifying over 200 volatile organic compounds in various effluvia, semi-quantitating about 100 of these and quantitating about 30 compounds.

Second, because of the facile transport of many compounds directly from the blood across the very thin membranes of the lungs into the lung air space, we can detect compounds of metabolic interest in the exhalant breath. Seasonal differences related to reduced food intake and correlated to seasonal reduction (and thus catabolism) of differential types of fat reserves have been noted in a population of wild bottlenose dolphins. Some populations of wild killer whales, *Orcinus orca*, selectively eat high fat content marine fishes such as herring and salmon; our breath sampling of these animals has demonstrated that individuals not getting adequate caloric intake exhibit an unusual ketogenic fingerprint of two elevated ketones in their breath; these elevations correlate with increased blood levels of these two ketones (Rasmussen & Hanson, in preparation). Studies on captive killer whales and circus elephants have shown the beneficial aspects of vigorous exercise as demonstrated by elevated components in breath indicative of healthy metabolic processes (Gerster, 1991). Our elephant breath studies, as reported in this article, demonstrate these metabolic reflectants, but also cross over into the next category of social signals.

Figure 2. Capturing exhalant breath from male manatee at Mote Marine Laboratory, Sarasota, FL. Dr. Debbie Colbert places the soft mask over the nostrils as the flaps open and lung air is expired, while Bets opens the valve on the evacuated sample bottle; exhalant air is thus captured for analysis. Manatees are elephants closest relatives.
Third, our studies on **social signals** have focused on the Asian and African elephants. The type of social chemical signal project possible is exemplified by musth and nonmusth studies of male Asian elephants. This mammal, an herbivore, may reduce his caloric intake during the musth state, and a weight loss of hundreds of kilos may occur. In the breath from male elephants in musth and those not in musth, clear-cut qualitative and quantitative differences distinguished that a spectrum of ketones from C4 through C12, as well as several related alcohols, are significantly elevated (Rasmussen et al., 1997; Rasmussen & Perrin, 1999; Rasmussen & Krishnamurthy, 2000; Rasmussen, Riddle & Krishnamurthy, 2002). This correlates well with elevations in serum lipase and triglycerides as well as changes in pH. Over a 10-year period (1994-2004) more than 100 samples from 10 male elephants have confirmed these results.

1. **Sampling technique: capture of breath volatiles**

   Our technique consists of catching the exhalant air emanating from the trunk into special stainless steel sampler bottles. These bottles are equipped with special “clean” valves that open instantly. The bottles are treated on their inner surface to be chemically inert. The samplers are under vacuum and will “suck” in 6 liters of air. The bottle is held near the tip of the trunk and when the elephant exhales, the valve is briefly opened, allowing the gathering of expired air into the sample bottle (Figure 3). About 5 openings/closures are sufficient to fill the bottle. Samples do not require refrigeration and can be stored indefinitely. Prior to analysis, the sample containers are pressurized with helium to 30 psi. Measurement of the percentage of carbon dioxide (CO₂) in each sample allowed the determination of sample quality and allowed standardization between samples. Normal air has less than 0.033±0.001% CO₂, whereas air released from mammalian lungs contains ~5% CO₂. Values less than 5% in a sample demonstrate the inclusion of non-lung expired air or non-tidal exhalant air. For quantitation the samples were standardized to 5% CO₂.

2. **Analytical gas chromatography/mass spectrometry of breath**

   The sampler bottles are designed to interface with a cryofocusing analytical method of gas chromatographic/mass spectrometry (gc/ms); this technique is designed to measure and identify trace gases. Excellent resolution, high sensitivity and quantitation are achieved for most classes of volatile compounds ranging from acetaldehyde through cyclic ketones up to molecular weights of about 225. Our technique has good sensitivity for aldehydes, acids, acetates, sulfides, alcohols and ketones with a detection limit of about 1 part per billion (Rasmussen and Perrin, 1999; Rasmussen, 2001).

   The analyses were conducted on a Hewlett-Packard 5890A gas chromatograph (GC) and a Hewlett-Packard 5970B mass spectrometer. The GC used a DB-1, 0.25-mm ID x 60 m x 1.0 µm film thickness,
polymethyl silicone-coated capillary column (J & W Scientific). The gas chromatograph oven was temperature programmed from –60 to 200°C at 4°C/min. The mass spectrometer was programmed for a mass scan of 33–300, which allowed for identification of compounds from C₃ through C₁₄. The conditions allowed quantitation as low as 0.10 ppbv. Compounds were identified using an NBS 75 K Hewlett-Packard Mass Spectrometer Chem Station library search and were manually rechecked with the NIST/EPA/NIH Mass Spectral Data Base Version 4.01. Internal standards of authentic compounds of measured concentration were employed for compounds of interest.

3. Concurrent confirmatory blood analyses
For our studies of elephant breath, we are currently confirming the reflection in exhalant breath of blood components as part of a long-term study of musth in captive Asian male elephants, using two analytical gc/ms techniques to examine concurrent samples of breath and blood, both collected from the same individual. Such confirmation, both qualitative and quantitative, is essential in demonstrating the reliability and utility of this non-invasive sampling method.

1. Health Issues
The initial step in this study was building a database of breath samples from healthy elephants. In our current database are captive and forest camp elephants of both species and sexes; this includes 15 Asian males, 8 Asian females, 5 African females and 6 African males. A number of these elephants, especially two African males at Riddle’s Elephant and Wildlife Sanctuary and two Asian males, at facilities in the U.S., have been repetitively sampled for 5-10 years.

GC-MS analyses of these “normal” samples identified about 125 volatile compounds including ketones from C₂-C₁₃, aldehydes from C₂-C₁₃, alcohols corresponding to these ketones and aldehydes, esters, low molecular weight acids, numerous hydrocarbons and sulfur compounds. Semi-quantitation (i.e. relative percentage in sample and in some instances actually concentrations per CO₂ levels) was possible with more than 30 of these identified compounds including most alcohols, aldehydes, hydrocarbons and ketones. In healthy elephants these ~30 volatile compounds are relatively constant with very little variation between species or sexes; some notable exceptions related to sex and hormonal status are briefly reviewed here.

In this section on health aspects we present two case studies that are non-conclusive but illustrate the medical potential of this type of non-invasive sampling.

The breath of a male African elephant [D] suffering from joint mobility problems demonstrated clearly elevated isoprene and pentane levels, potentially indicative of respectively fat and muscle breakdown. Samples taken from this same elephant of temporal gland secretions (TGS), which are a reflection of both blood and apocrine secretions, also demonstrated elevated isoprene and pentane. In the breath, neither sulfur nor the spectrum of alcohols were elevated as seen in certain bacterial or fungal infections, but higher than normal acetic acid and two other acids were seen: 3-methyl butanoic acid and hexanoic acid. The latter can be released by Shigella, a non-motile aerobic bacteria species, as well as by

Results
Elephants prove to be ideal subjects for capturing exhalant breath. With few exceptions, the carbon dioxide percentage was between 3.5-4.5%, indicating that well over 50% of the air captured in the sample bottles was exhalant air. Unlike sampling with humans, we could not “tell” the elephants to begin exhalations, so logistically we did not obtain as high a percent of true “tidal air” as possible with humans, as evidenced by the 3.5-4.5% CO₂ not 5%. However, because of the dominance of trunk breathing, the exhalant elephant sample was a more complete reflection of lung air than mammals who mouth breathe, although less than marine mammals with totally separate respiratory and digestive systems.
Aspergillus, a fungus (Brooks et al., 1985; Brown et al., 1996). High acetic acid is released by Clostridium spp, but also by common lactic acid bacteria (Stadtman et al., 1972; Tracey & Britz, 1989). Extremely high levels of furan (27%) were observed. Possibly these are diet related, coming from dihydroxy isopentyl substituents of Rutaceae or Umbelliferae plants, which are aromatic trees and shrubs. More likely these extremely elevated amounts are bacterial or fungal related.

A second recent example involves another male African elephant [S] that seemed to have increased trunk drainage and appeared somewhat uncomfortable. Five acids, but not furan, were elevated and these were: acetic acid, propionic acid, butanoic acid, pentanoic acid and hexanoic acid. Again possible bacterial links exist to Clostridium spp or to Shigella spp. In addition, an apparently killer whale-specific Pseudomonas spp. releases hexanoic acid (Rasmussen & Walsh, unpublished); and other Pseudomonas spp. release other acids (Zechman & Labows, 1985; Nieder & Shapiro, 1975). Staphylococci will also metabolize glucose to acetic acid (Seidl & Schleifer, 1978). Control breath samples from a companion male African elephant [T] taken on the same day, and previous samples from [S] did not demonstrate these high acids. In the Discussion section we present more definitive correlations with specific bacteria and fungi in marine mammals that will strengthen the potential of these analyses in elephants.

2. Metabolic Reflections
The breath as a reflection of the internal circulatory system is clearly illustrated by changes in volatiles during pregnancy in female Asian elephants and during musth in male Asian male elephants. Although three of our previous publications (Rasmussen, 1998; Rasmussen & Krishnamurthy, 2000, 2001) and several talks have discussed these situations, a brief review is relevant as this topic links to health. During musth many ketones in

breath elevate (especially 2-butanone, 2 and 3-pentanone, cyclopentanone and cyclohexanone and, for older males, 2-nonanone) (Rasmussen, 1998; Rasmussen, Riddle & Krishnamurthy, 2002). At times a concurrent elevation of 2-butanol (with high 2-butanone) is observed, perhaps indicative of Staphylococcus xylosus or S. equorum. However, as elevated levels of a methyl branched ketone, 3-methyl-1-butanol, are not observed more likely 2-butanone degrades via a well-described stereo specific alcohol dehydrogenase to 2-butanol (Peng et al., 1995).
Interestingly, both in male elephants in musth and in near term pregnant female elephants, another breath (and blood) ketone, 6-methyl-5-hepten-2-one, which is linked to an estrogen influenced metabolic shunt, and isoprene both elevate (Rasmussen & Krishnamurthy, 2000, 2001). There is a suggested relationship between the concentration of the latter compound and the levels of fatty acids, cholesterol and steroids (Deneris et al., 1985).

3. Social Signals
Much of our research has focused on chemical compounds which function as elephant social signals. Again in the somewhat redundant and honest chemical signaling system of elephants, many communicative compounds are present in the blood and excreted or secreted into the urine, breath or TGS. The compound Z-7-dodecenyl acetate is detected in blood and released in urine. Frontalin, indicative of musth in older males, is an especially redundant system present in blood, TGS, urine and breath. The chemical identification of other social signals is a future priority, as their identity will help us understand in depth and precision the functioning of elephant society. For example, we already know from studies at several captive elephant facilities in the U.S., that male Asian elephants in musth will blow at each other under doors and across fences, strongly suggesting a transfer of chemical signals between individuals (Figure 4). Additional studies may reveal this behavior by elephants during other situations and we are interested in identifying what which specific compounds are being utilized by the elephants.
Discussion and Reflective Thoughts

The basic message of this report is that we have developed a simple and non-invasive technique to study several aspects of elephant health, metabolism and society. It is easy to collect exhalant elephant breath, either 1. by simply placing the orifice of the sample bottle under vacuum directly at the end of the trunk, or 2. with varying lengths of tubing attaching a funnel to the sample bottle to allow collection from difficult elephants, or 3. even setting the sample bottle at the opening of a hole in an enclosure wall and waiting for the elephant to exhale into the hole. Significantly, our data shows that the breath from most elephants, regardless of species, looks generally similar. Yet observed differences are striking, both qualitatively and quantitatively. Importantly, from a health perspective the majority of elephants had “clean” breath. Two examples are given in this article of nonmusth African males in whom specific elevations of a few compounds were observed. We offer suggestions of possible correlations to bacterial or fungal infections. While currently there are no developed human breath tests for a bacterial disease such as tuberculosis, we would like to explore this possibility in elephants, both because of their unusual respiratory system and our ability to capture high quality exhalant breath samples quickly and easily, thereby enhancing the potential for non-invasive diagnosis and medical management.

Interestingly, in the elephant breath samples analyzed to date, we have not seen high concentrations of alcohols similar to those seen in a foot abscess of an elephant and in unhealthy marine mammals. For the latter, we initially obtained baseline data of healthy breath samples from numerous captive killer whales. We had a two-fold purpose in obtaining samples of killer whale breath: 1. early diagnosis of respiratory infections – either bacterial or fungal, and 2. assessment of the beneficial metabolic effects of moderate and vigorous exercise. Concentrations of more than 30 identified compounds including alcohols, aldehydes, esters, hydrocarbons, ketones and sulfur compounds were tabulated. Exercise affected the levels of pentane, selected hydrocarbons and several ketones. When breath samples were obtained from a whale with a known respiratory infection, elevated levels of selected compounds, especially alcohols, were revealed. Concurrent culture studies of killer whale respiratory bacterial infections did not demonstrate high levels of ketones; rather a set of alcohols including ethanol, 1-butanol, 2-propanol, 3-buten-1-ol, 3-methyl-1-butanol (Staphylococcus xylosus) were elevated. Studies were expanded to other captive marine mammals including manatees, dwarf sperm whales, pygmy sperm whales and rough toothed dolphins, and wild marine mammals including fur seals and bottlenose dolphins. From the latter, concentrations of more than 75 identified compounds including low molecular weight acids as well as an expanded repertoire of alcohols, aldehydes, esters, hydrocarbons, ketones and sulfur compounds were assessed. In captive killer whales and in wild dolphins with respiratory infections, ethanol, 2-propanol, 1-butanol and other alcohols were elevated. Several types of bacterial infections release substantial amounts of 2-propanol. Elevated 1-butanol may be a product of bacteria such as Pseudomonas spp. or the actinomycetes, Streptomyes spp or the fungi Stachybotrys chartarum. Although 2-butanol elevations may be resultant from high 2-butanone levels, concurrent elevation of 2-butanol along with high 2-butanone may be indicative of the presence of bacteria (Staphylococcus xylosus or S. equorum). A methyl branched alcohol, 3-methyl-1-butanol, is more definitively indicative of the presence of Staphylococci spp. (Peng et al., 1995).

Metabolic events such as muscle breakdown or liposis may be indicated by chemical compounds detected in exhalant breath. Chemical compounds detected in exhalant breath may indicate metabolic events such as muscle breakdown or liposis. For example, high levels of 2-butanone detected in the breath of musth male elephants may be an indica-
tor of lipid peroxidation. Oxidative stress in biological systems can result in membrane breakdown; so reactive oxygen can cause oxidation of membrane lipids resulting in the formation of lipid peroxidation products such as malondialdehyde. This aldehyde can originate from poly-unsaturated (3 or more double bonds) fatty acids in membranes and is the most widely used index of lipid peroxidation. The compound 2-butanone is a common ketone elevated when fat metabolism predominates, and may be produced in mammalian cells from malondialdehyde and thus indicates lipid peroxidation. Concurrent elevated pentane may further implicate this metabolic route.

Data from breath samples of marine mammals in specified conditions reinforce such metabolic effects seen with male elephants in musth. Ketones that may be elevated in musth males include 2-butanone, 3-pentanone (and other pentanones) and 2-nonanone. Similarly, the breath of a lactating, but non-feeding wild fur seal was characterized by high levels of 3-pentanone and moderately high levels of 2-butanone. Similarly, high levels of 3-pentanone and moderately high levels of 2-butanone characterized the breath of a lactating, but non-feeding wild fur seal. Late term pregnant Asian elephants demonstrate high isoprene and sometimes elevated ketones like 6-methyl-5-hepten-2-one. Interestingly the breath of a wild male fur seal demonstrated high levels of 3 different ketones: 2-butanone, acetophenone and 6-methyl-5-hepten-2-one. The latter ketone is under hormonal control and may be indicative of a mevalonate shunt shift from synthesis of sterols to ketones.

Breath also contains relevant social signals. As the focus of much of our efforts has been on the influence of chemical signals and pheromones from the urine and temporal glands on elephant societal interactions (Rasmussen & Krishnamurthy, 2002; Rasmussen, Riddle & Krishnamurthy, 2002), and since our research has demonstrated a correlation between blood constituents and TGS components, an important message of this report is that many of these same chemical communicator molecules are excreted to the outside environment through the breath. What makes these signals special is they are more ephemeral than urinary or TGS secretions. They are carried in the air, probably in part on aerosols, and are usually rather volatile. They diffuse rapidly in the air medium and are subject to photooxidation. Thus their message is immediate, often close range and non-persistent, thus and may be individual-to-individual communication. Future close studies on such molecules and their meanings to elephants should be revealing.

References


