Collection and analysis of breath and breath condensate exhaled by feral pigeons (Columba livia) and chickens (Gallus domesticus)

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Abstract

Samples of exhaled breath and breath condensate were collected from 20 feral pigeons (Columba livia) while they were anaesthetised and intubated, and when they were kept unanaesthetised in an acrylic box. Samples were also collected from six chickens (Gallus domesticus) while they were kept in an acrylic box. The samples were analysed for pH, nitric oxide (NO), hydrogen peroxide and leukotriene B4. The volume of condensate collected from the pigeons was independent of bodyweight and significantly more (1·66 [0·64] ml/kg) was obtained while they were in the acrylic box than when they were intubated (0·87 [0·32] ml/kg). The mean volume collected from the chickens was 0·15 (0·06) ml/kg. Cooled samples had higher concentrations of NO than uncooled samples. The pH of the samples of condensate collected from birds in the acrylic box were significantly higher (7·9 [0·3]) than those from the intubated birds (5·3 [0·1]), and samples from the chickens had significantly higher pH values than samples from the pigeons (8·2 [0·2] v 7·9 [0·3]).
Original research

Collection and analysis of exhaled breath and exhaled breath condensates in feral pigeons (*Columba livia*) and chickens (*Gallus domesticus*)

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Summary

Twenty feral pigeons (*Columba livia*) and six chickens (*Gallus domesticus*) underwent collection of exhaled breath (EB) and exhaled breath condensate (EBC). Collection took place in anaesthetised, intubated pigeons and in awake pigeons and chickens in an acrylic box. Samples were analysed for pH, nitric oxide (NO), hydrogen peroxide, and leucotriene B4, which in mammals are indicators for inflammatory disease. The amount of EBC collected from pigeons was independent of body weight and the EBC collected from chickens (mean=0.15±0.06 ml/kg body mass) were significantly smaller than from the pigeons. The amount of EBC obtained from pigeons in the acrylic box (mean=1.66±0.64 ml/kg body mass) was significantly more than in intubated pigeons (mean=0.87±0.32 ml/kg body mass). Cooled (~ 4°C) samples resulted in higher NO values than uncooled samples and birds appear to have higher NO values than mammals. The pH measurements from the acrylic box EBC were significantly higher (7.9 ± 0.3 vs. 5.3 ± 0.1) than from intubated birds and chickens had significantly higher pH values compared to the pigeons (8.2 ± 0.2 vs. 7.9 ± 0.3). The analysis of EB and EBC has the potential for a novel and non-invasive approach for the detection of respiratory tract disease in birds.

Key words: avian medicine, respiratory tract disease, diagnosis
Diseases of the respiratory tract in birds are common causes of morbidity and mortality (Lawton 1999; Tully 1995). Infections especially mycoses caused by *Aspergillus* spp. are frequently diagnosed in post mortem examinations. Intra vitam diagnosis of respiratory tract disease in avian species remains a challenge. Often diagnosis is only made when the disease has progressed to a stage where prognosis is guarded to bad. Radiology, computer tomography, and tracheopulmonic lavage are important diagnostic tools. However, especially in critically ill birds these methods may pose a risk as they often require general anaesthesia. Other diagnostic methods such as endoscopy of the trachea or the coelomic cavity are invasive and moreover require general anaesthesia.

Whereas the last years have brought several improvements in avian medicine regarding the therapy, especially of aspergilliosis, little progress has been made regarding the diagnosis of respiratory tract diseases in birds in general. Recent developments in human medicine are therefore of special interest. Analysis of exhaled breath (EB) and exhaled breath condensates (EBC) plays an important role in the diagnosis of many human lung diseases including asthma, chronic obstructive pulmonary diseases (COPD), and bronchiectasis (Kharitonov and Barnes 2001). Especially in neonates and children the advantages of breath analysis are obvious, as this method allows painless collection, frequent repeating, and follow up of the response to the therapy. The exhaled EB consists of a gaseous phase, which contains volatile compounds such as nitric oxide (NO) and carbon monoxide. The EBC contains many biomarkers of oxidative stress. Inflammatory markers which are currently investigated in EBC are hydrogen peroxide (*H*₂*O*₂), leucotriene B₄ (LTB₄), prostaglandins, and histamines. These mediators are released from the airway lining fluid, carried up by exhaled breath, and subsequently collected by condensation of the exhalate by breathing into a cooled tube. In animals analysis of EB and EBC is currently investigated at research level. Studies have been carried out with several species of mammals as reviewed by Zollinger et al. (2006). The
main conclusion of this review was, that EB and EBC can be obtained from a wide variety of species, but the authors pointed out, that for EB and EBC analysis to become a diagnostic tool, further investigation are necessary to evaluate the effect of collection variables, such as sampling rate, sampling method, and storage temperature and to propose a standardized collection protocol for each species.

In avian species to date no data has been published regarding EB and EBC collection and analysis. In view of the frequency of respiratory tract disease and the risk of stress in birds the analysis of EB and EBC appears to be a promising method.

The aim of the present study was to develop a method for EB and EBC collection in two model species of birds, feral pigeons (*Columba livia*) and domestic chickens (*Gallus domesticus*), and to investigate if in these species inflammatory markers such as NO, H2O2, and LTB4 as well as pH could be measured.

### Material and methods

The present study involved 20 feral pigeons and six domestic chickens. All animals were clinically healthy. Pigeons were adult and their body weight (BW) ranged between 340 and 450 g. Chickens were six month old and weighed between 2.8 and 3.3 kg. Prior to the start of the study all birds underwent a general health examination including haematology, blood chemistry, faecal parasitology and faecal bacteriology. Both species were kept in groups in outdoor aviaries. Diet consisted of a commercial avian pelleted diet and water, which were offered ad libitum.

The study was evaluated and approved by the animal care and use committee of the Canton of Zurich, Switzerland (Permit No. 46/2005).

Two different methods of exhaled breath collection were investigated.

In trial 1 ten pigeons under general anaesthesia were intubated with a Cole’s endotracheal tube (Cook® Veterinary Products, Medical Solution, Steinhausen, Switzerland) which was
connected to a non-rebreathing valve (Model 2-W, Hugo Sachs Electronic-Harvard Apparatus GmbH, March-Hugstetten, Germany). The setup is graphically displayed in Figure 1. Two polypropylene tubes (inner diameter 6 mm and outer diameter 7 mm) were connected to the valve. The inspiratory tube was connected to a 10 l respiratory bag (Hans Rudolf Inc., Kansas City, USA) filled with synthetic air (80% N and 20% O2). The expiratory tube was fitted with a three-way switch, which allowed the separate collection of both EB. The EB was collected over a 18G needle into a 6 ml Vacutainer (Vacuette®, Greiner Vacuette Bio-One, St. Gallen, Switzerland). For the collection of EBC the expiratory tube following the three-way switch was led through an ice-water bath (~4°C). The EBC was collected into an Eppendorf vial after the ice-water bath. At the end of the sampling the expiratory polypropylene tube was cut into smaller pieces and subjected to centrifugation at 4000 rpm and a constant temperature of 4°C (Centrifuge 5810 R, Eppendorf Vertrieb Deutschland GmbH, Wesselwing-Berzdorf, Germany) to collect additional material.

Anaesthesia of the pigeons in trial 1 was induced by intramuscular application of 0.2 mg/kg BW medetomidine hydrochloride (Domitor®, Dr. E. Gräub, Bern Switzerland), 100 mg/kg BW ketamine (Narketan®10, Vétoquinol AG, Bern, Switzerland), and 0.2 mg/kg BW butorphanol (Morphasol®, Vétoquinol AG, Bern, Switzerland). Repeated doses were given as necessary for a total anaesthesia time of up to three hours. Medetomidine hydrochloride was reversed with atipamezole hydrochloride (Antisedan®, Dr. E. Gräub, Bern Switzerland). During anaesthesia the pigeons were kept in a ventral position on a heat blanket. Body temperature was constantly measured via a probe inserted into the crop (Cooper Instrument Corporation, Middlefield, USA). Constant intravenous lactated ringer solution was applied through the ulnar vein at a rate of 3 ml/h through an infusion pump (Injectomat® Fresenius, Stans, Switzerland).
Within 20 min after sample collection, pH of the breath condensate was measured (691 pH Meter, Metrohm, Herisau, Switzerland) and subsequently the sample was immediately frozen at minus 80°C until further processing for LTB4 and H2O2 analysis.

In trial 2 animals were maintained for up to two hours in an acrylic glass box. The study was performed with ten pigeons and six chickens. The setup is graphically displayed in figure 2. The measurements for the box for pigeons were 0.25 x 0.25 x 0.25 m and for the chicken the measurements were 0.5 x 0.5 x 0.5 m. The box was tightly fitted to the surface of the table with tape and was equipped with a thermo- and hygrometer. Access for synthetic air was through an inlet in the top of the box, an outlet was near the floor. The flow-rate of synthetic air for the pigeons was 0.9 l/min and for the chickens it was 6.6 l/min. For sample collection the setup regarding the cooling system and the three-way switch was identical to trial 1.

Collection of EB was always performed 90 min after introducing the bird into the box.

Samples for the analysis of NO were divided into two groups. One group was stored on ice until analysis, which took place within one hour of collection. The other group was kept at ambient temperature. Measurement of NO concentration was carried out by chemiluminescence (Analysator CLB 88 sp, ECO MEDICS AG, Dürnten, Switzerland) with a range of detection between 0.1 and 5000 ppb.

Prior to H2O2 analysis condensate samples were left to defrost at ambient temperature. A volume of 300 µl of the condensate was mixed with an identical volume of phosphate buffer. Subsequently H2O2 was measured with a biosensor (EcoCheck H2O2, FILP Lungen- und Thoraxdiagnostik GmbH, Berlin-Buch, Germany). According to the manufacturer the limit of detection was at 15 nmol/l.
For the measurement of LTB4 a commercial enzyme immunoassay was used (Leukotriene B4 EIA kit, Cayman Chemical Company, Ann Arbor, Michigan, USA). Analysis was performed according to the manufacturer’s instructions.

All statistical analyses were performed using a software package for personal computers (Statistika™ 7.1, StatSoft®, U.S.A.). Statistical significance was set at p < 0.05. The objective of the analyses presented in this paper was to determine, whether there was a difference in analyzed parameters between the two trials and whether there was a relationship between analyzed parameters and sampling and storage methods. Means are given with standard deviation.

While the influence of storage methods and investigated species on measured parameters were analyzed using the paired t-test, the two sample test was used for evaluating the influence of sampling methods and species on EBC and pH. It was assumed that data from each sample were normally distributed and that each sample had the same variance, the latter were tested by Kolmogorov-Smirnov test and Hartley F\text{max}-Test, respectively. If the assumptions were not satisfied or sample number was below five, the alternative nonparametric Mann-Whitney U- (U) or Wilcoxon Matched pairs (Z) tests were performed. Pearson product-moment correlation coefficient was calculated for the relationships between EBC and body mass.

Results

All animals appeared clinically healthy during the entire study, with the exception of pigeon no. 7, which died unexpectedly anaesthesia. Post-mortem investigation of this bird revealed a fresh haemorrhage in the coelomic cavity, there were no external signs of trauma and the aetiology for the haemorrhage could not be determined.
Regarding the storage of NO on ice or at ambient temperature, there was a significant
difference with cooled samples having higher values (99.6 ± 37.0 ppb vs. 80.7 ± 33.4 ppb; t =
7.92, p < 0.001) figure 3.

In trial 1, 0.17 – 0.46 ml of EBC could be collected within three hours of general anaesthesia.
Table 1 summarises the values obtained. In pigeon 1 – 4 the amount of EBC was not
measured and 0.2 – 0.35 ml that were used for the analysis. For the comparison of EBC
volumes with this species these ranges were therefore not included. Cooled and uncooled NO-
values from pigeon 1 - 4 were significantly lower than from pigeons 5 – 10 (cooled: 85.1 ±
18.1 ppb vs. 146.8 ± 39.6 ppb; U = 0.00, p=0.011 and uncooled: 60.8 ± 22.9 ppb vs. 126.5 ±
33.2 ppb; U = 0.00, p=0.011). The \( \text{H}_2\text{O}_2 \) values in the incubated pigeons were all below the
limits of detection. Leucotriene B4 were close or below the detection limit, with the exception
of pigeon 3 which had an apparently very high concentration.

Results from trial 2 are summarized in tables 2 and 3. During the two hours, that the birds
spent in the acrylic glass box, no signs of stress were observed. The relation of box to body
surface was approximately double for the pigeon than for the chicken (Tab. 4).
The amount of EBC obtained from pigeons in the acrylic box (mean=1.66±0.64 ml/kg) was
significantly more (t=-2.83, p=0.01) than in intubated pigeons (mean=0.87±0.32 ml/kg).
The amount of EBC in the intubated pigeons (n=6, 0.40-1.26 ml/kg) and pigeons maintained
in an acrylic box (n=10, 1.03-2.92 ml/kg) was independent of body weight (F=0.13, p>0.05;
F=-0.37, p>0.5). The amount of EBC collected from chickens (mean=0.15±0.06 ml/kg) were
significantly smaller (t=5.75, p<0.001) than from the pigeons (1.66±0.64 ml/kg). During the
three hour period temperature (Z=2.02, p=0.04) and relative humidity (Z=2.52, p=0.01) in the
acrylic box changed significantly with pigeons, while no significant changes were noted with
chickens (p>0.05). Uncooled NO concentrations of EB in chickens and pigeons kept in the
box were lower than cooled NO concentrations but in contrast to trial 1 the difference was not significant (cooled: 77.7 ± 24.5 ppb vs. 89 ± 19.7 ppb; t=1.02, p=0.33, uncooled: 64.1 ± 18.6 ppb vs. 70 ± 13.3 ppb; t=0.73, p=0.48). In contrast to trial 1, H$_2$O$_2$ could be measured in all but one pigeon in trial 2. Concentrations were in the range of 0.04 – 2.26 µmol/l. The H$_2$O$_2$ concentration in pigeons and chickens appear to be in the same range, but no statistical analysis was performed since only three out of six chickens produced large enough EBC volumes for the analysis of H$_2$O$_2$. The LTB4 values were below detection level in all birds.

The pH values were significantly higher in trial 2 compared to trial 1 (7.9 ± 0.3 vs. 5.3 ± 0.1; t=17.68, p<0.001) and chickens had significantly higher pH values compared to the pigeons (8.2 ± 0.2 vs. 7.9 ± 0.3; t=2.24, p=0.04).

**Discussion**

The present study demonstrated, that EB and EBC could be collected in two species of birds, with a body weight range between 0.3 and 3 kg. Collection of EBC from pigeons kept in an acrylic glass box was approximately 2.5 times higher than from intubated birds. Due to its less stressful approach, the collection method in the box appears particularly useful for birds in a clinical setting, especially in patients with a potential ongoing disease process. However, compared to the collection of EB and EBC in intubated birds, the collection from the box carries certain risks of sample contamination, through excrements and cutaneous evaporative water loss. The latter has been described in pigeons by Webster and King (1987) and led to a significant increase of relative humidity, which was observed in the present study as well. Differences in cutaneous water loss might explain why pigeons also produced overall larger EBC volumes than chickens. Alternatively, the difference in EBC volumes between pigeons and chickens could also be explained by the fact, that chickens had a smaller box than the
pigeons compared to their body surface (Tab. 4) but a higher air flow. These factors might have resulted in relative humidity in chickens that was not significant.

It could be demonstrated in the chicken and the pigeon, that the volume of EBC was not correlated to body mass. This is in contrast to calves, where a correlation between body mass and EBC volume was found (Reinhold and others 2000; Reinhold and others 1999). An explanation for this could be, that the respective body mass ranges in the two species was too small in relation to the range of EBC. This assumption is corroborated by the observation that chickens 1 and 5, which were of similar body mass, had EBC volumes of 1.24 and 0.40 ml, respectively. Pigeons 13, 14, and 15 had the same body mass but respective EBC volumes of 0.96, 0.55, and 0.40 ml were collected. It is also interesting to note, that pigeons produced approximately four times less EBC volume per kilogramm body mass compared to cat, dog, calf, and horse (Deaton and others 2001; Deaton and others 2004; Pietra and others 2003; Reinhold and others 2000; Reinhold and others 1999; Sparkes and others 2004; Wyse and others 2004).

In the present study the parameters to be analysed (H$_2$O$_2$, LTB4, NO, and pH) were chosen because they have been found to be important indicators of inflammation in human medicine. In veterinary medicine research, breath analyses have been performed in different mammalian species. Most studies were performed with clinically healthy animals. Exceptions are studies in calves and horses. In calves intratracheal inoculation with Pasteurella multocida resulted in a significant elevation of LTB4 in the EBC within three days (Reinhold and others 2000; Reinhold and others 1999). However, values were still within the reference range of healthy calves. In horses with inflammatory disease of the lower respiratory tract it was found that H$_2$O$_2$ in the EBC was significantly increased (Deaton and others 2002).
We could show, that cooled (~ 4°C) samples resulted in higher NO values than uncooled samples. In pigeons this difference was significant. This is in contrast to Bodini et al. (2003) that found, that storage of NO samples for 9 h at 4°C, 21°C, or 37°C had no effect on concentration. In their study NO samples were collected in mylar ballons, which are non-reactive. In our study samples were collected in vacutainers, which in theory should be airtight. A possible inflow of air and the occurrence of the chemical reaction 2 NO + O₂ = 2 NO₂ cannot be completely ruled out and this could be an explanation for the difference in values. It is therefore recommended that unless online NO measurement is performed, samples should be stored at 4°C and analysis should be performed without delay.

In the intubated pigeons a significant difference of the NO concentrations was found between the animals 1 - 4 and 5 - 10, with the values of the second group being higher. Since all animals were kept in outdoor aviaries, a possible explanation could be, that the animals with the higher values were investigated during winter months, whereas pigeons 1 - 4 were investigated during summer. The higher fine particulate concentration in the air during winter has been observed to cause respiratory tract disease including bronchitis or asthma in humans (Gillissen and others 2006; Lin and others 2002; Thompson and others 2001). A further type of asthma induced by cold air has also been observed by one of the authors (MH) to induce increased NO-values in EBC in humans. To corroborate the hypothesis of higher values in pigeons in winter vs. summer, further studies are needed investigating the same animals during different seasons.

The NO concentrations were similar between pigeons and chickens, and in pigeons in the box NO concentrations were lower than in intubated pigeons of trial 1, but the difference was not statistically significant. Nevertheless, NO values from intubated pigeons can not be compared with values from the pigeons in the box, due to the impact of airflow in the box, which influences especially NO in EBC collected from animals kept in a box. It was shown by Weicker et al. (2001) in mice in a similar setup as ours, that the concentration of NO in the
EBC is inversely correlated to the airflow into the box. Furthermore the NO analysed from intubated animals is considered to truly reflect the situation in the lower respiratory tract. EBC collected from animals kept in a box or from a face mask have been shown to be different because of the NO from paranasal sinuses (Lewandowski and others 1998). In birds the respiratory tract anatomy is different from mammals, due to the absence of alveoli, comparatively smaller lungs, and the presence of large airsacs, which act as bellows. Additionally, most bird species have a large paranasal sinus system. The respiratory tract in birds is comparatively larger than in mammals, which results in a larger surface, that can produce NO (O'Malley 2005; Vollmerhaus and Sinowatz 1992). This might explain, why in this study NO values were in the range of 34 – 87 ppb, whereas in mammals only rhesus monkeys (Macaca mulatta) had concentrations above 30 ppb (Zollinger and others 2006). Regarding the anatomical differences it should also be noted that, other sources outside the respiratory tract may influence NO values, such as bacteria, or NO produced in the stomach (Bernareggi and Cremona 1999).

For the analysis of \( \text{H}_2\text{O}_2 \) it was found, that the necessary sample volume of 300 µl was prohibitively large and allowed the measurement of \( \text{H}_2\text{O}_2 \) only in the animals kept in the acrylic glass box. It was tried to dilute the sample by adding 250 µl of aqua bidestillata to a 50 µl aliquot. But the measured values were similar to measuring only the phosphate buffer. Nevertheless the values measured in the pigeons and chickens kept in the box are in the same range as the values reported for mammals and humans (Zollinger and others 2006).

In the present study it was found, that LTB4 values from pigeon were low compared to mammalian animals (Zollinger and others 2006). The only exception was pigeon 3, which had an LTB4 concentration of 25.7 pg/ml. This animal was re-examined 14 days later and then had a value of 3.0 pg/ml. It therefore appears, that the elevated first value was likely caused...
by an analytical error, although it can not be excluded, that the animal had suffered from a
pathological process, which in the meantime had healed. This appears less likely, since the
other values, pH and NO, were not different from the other pigeons.

The LTB4 analysis was always performed after the H2O2 measurement. Between the two
analyses, samples were stored at 4°C for up to four weeks. It might be possible that during
this time concentrations decreased due to degradation processes. This could explain, why
none of the birds sampled in the box had detectable LTB4 values. These samples were
analysed after a longer period than samples from trial 1. In future studies we therefore
recommend to freeze separate samples, which can be analysed immediately after defreezing.

The pH of EBC in the pigeons in the box was 2 to 3 log higher than in the intubated pigeons.
As for the NO, a possible difference could be the influence of evaporation outside the
respiratory tract. Furthermore, additional volatile components such as CO₂ or ammonia may
originate from feather dust or from faeces.

In summary, we could show, that EB and EBC can successfully be collected and parameters
can be determined in chickens and pigeons, which in mammals are considered indicators for
inflammation. The interpretation of the results and comparison with other studies is
challenging due to the wide range of the concentration of inflammatory markers and the
different methods used. For further development of EB and EBC analysis it is therefore very
important, that the sample collection procedure has to be standardized with regard to the
sampling technique, the surface of the sample collection tube, the temperature of fluid around
the condensing chamber, the ambient humidity, the respiratory rate, and the storage of EB and
EBC. When a Plexiglas chamber technique is used, the flow rate plays an important role, too,
because this can have an effect on NO concentration.
Further systematic studies with additional species, such as psittacines or birds of prey and birds with respiratory tract pathology are needed to determine the diagnostic application of EB and EBC analysis in birds. The technical equipment using a box as described in the present study is inexpensive and easy to install. If pH could be detected as a diagnostic parameter this would be very useful since analysis could be easily performed in a clinic setting. The other parameters NO, LTB4, and H2O2 might prove to be less useful, since analysis would need to be carried out in a specialised laboratory. In the future development of on-line NO measurements in human medicine might however produce affordable online measurement equipment for veterinary practice.

Acknowledgements

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KHARITONOV, S. A. & BARNES, P. J. (2001) Exhaled markers of inflammation. Current Opinion in Allergy and Clinical Immunology 1, 217-224
TABLE 1: Analysis of exhaled breath (nitrous oxide) and exhaled breath condensates (H$_2$O$_2$, pH, and leucotriene B4) in intubated clinically healthy feral pigeons (*Columba livia*) under general injectable anaesthesia. Cooled nitrous oxide samples were stored on ice until analysis, uncooled samples were stored at room temperature.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Body mass</th>
<th>Exhaled breath condensate</th>
<th>Nitrous oxide</th>
<th>Leucotriene B4</th>
<th>H$_2$O$_2$</th>
<th>pH</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>g</td>
<td>cooled ppb</td>
<td>pg/ml</td>
<td>µmol/l</td>
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<tr>
<td>Pigeon 1</td>
<td>339</td>
<td>NM</td>
<td>88.0</td>
<td>7.3</td>
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<tr>
<td>Pigeon 2</td>
<td>380</td>
<td>NM</td>
<td>105.0</td>
<td>5.3</td>
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<td>4.3</td>
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<tr>
<td>Pigeon 3</td>
<td>340</td>
<td>NM</td>
<td>86.4</td>
<td>25.7</td>
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<td>5.3</td>
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<td>Pigeon 4</td>
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<td>NM</td>
<td>61.1</td>
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<td>136.9</td>
<td>&lt;detection limits</td>
<td>detection limits</td>
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NM: not measured
### TABLE 2: Analysis of exhaled breath (nitrous oxide) and exhaled breath condensates (H₂O₂, pH, and leucotriene B₄) in clinically healthy feral pigeons (*Columba livia*) maintained in an acrylic box. Cooled nitrous oxide samples were stored on ice until analysis, uncooled samples were stored at room temperature.

<table>
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<tr>
<th>Unit</th>
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<th>Pigeon 12</th>
<th>Pigeon 13</th>
<th>Pigeon 14</th>
<th>Pigeon 15</th>
<th>Pigeon 16</th>
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<th>Pigeon 18</th>
<th>Pigeon 19</th>
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<td><strong>Exhaled breath condensate</strong></td>
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<td>0.96</td>
<td>0.55</td>
<td>0.40</td>
<td>0.47</td>
<td>0.76</td>
<td>0.79</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Temperature in box (start / end)</strong></td>
<td>°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>n.a.</td>
<td>n.a.</td>
<td>23 / 25</td>
<td>19 / 22</td>
<td>23 / 24</td>
<td>21 / 22</td>
<td>22 / 22</td>
<td>22 / 22</td>
<td>22 / 22</td>
</tr>
<tr>
<td><strong>Relative humidity (start / end)</strong></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>n.a.</td>
<td>n.a.</td>
<td>45 / 76</td>
<td>45 / 60</td>
<td>45 / 60</td>
<td>45 / 55</td>
<td>32 / 55</td>
<td>20 / 35</td>
<td>20 / 26</td>
</tr>
<tr>
<td><strong>Nitrous oxide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cooled ppb</td>
<td></td>
<td>95.5</td>
<td>78.7</td>
<td>n.a.</td>
<td>49.7</td>
<td>67.5</td>
<td>102.1</td>
<td>102.1</td>
<td>105.6</td>
<td>97.4</td>
</tr>
<tr>
<td>uncooled ppb</td>
<td></td>
<td>70.1</td>
<td>61.2</td>
<td>n.a.</td>
<td>43.7</td>
<td>61.1</td>
<td>87.9</td>
<td>82.3</td>
<td>79.0</td>
<td>72.9</td>
</tr>
<tr>
<td><strong>Leucotriene B₄</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pg/ml</td>
<td></td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H₂O₂</strong></td>
<td>µmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.16</td>
<td>&lt; detection limits</td>
<td>0.18</td>
<td>0.22</td>
<td>0.45</td>
<td>0.04</td>
<td>0.20</td>
<td>0.62</td>
<td>2.26</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.6</td>
<td>7.3</td>
<td>7.7</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
<td>7.7</td>
<td>7.8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

n.a. not analysed
TABLE 3: Analysis of exhaled breath (nitrous oxide) and breath condensates (H₂O₂, pH, and leucotriene B₄) in clinically healthy domestic chickens (*Gallus domesticus*) maintained in an acrylic box. Cooled nitrous oxide samples were stored on ice until analysis, uncooled samples were stored at room temperature.

<table>
<thead>
<tr>
<th>Unit</th>
<th>Chicken 1</th>
<th>Chicken 2</th>
<th>Chicken 3</th>
<th>Chicken 4</th>
<th>Chicken 5</th>
<th>Chicken 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass g</td>
<td>2850</td>
<td>3020</td>
<td>2970</td>
<td>3320</td>
<td>2880</td>
<td>3270</td>
</tr>
<tr>
<td>Exhaled breath condensate ml</td>
<td>1.24</td>
<td>0.58</td>
<td>0.96</td>
<td>0.55</td>
<td>0.40</td>
<td>0.47</td>
</tr>
<tr>
<td>Temperature in box (start / end) °C</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>21 / 22</td>
<td>21 / 21</td>
<td>21 / 21</td>
</tr>
<tr>
<td>Relative humidity (start / end) %</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>20 / 27</td>
<td>19 / 23</td>
<td>20 / 21</td>
</tr>
<tr>
<td>Nitrous oxide cooled ppb</td>
<td>66.5</td>
<td>35.2</td>
<td>98.8</td>
<td>76.8</td>
<td>88.9</td>
<td>99.9</td>
</tr>
<tr>
<td>Nitrous oxide uncooled ppb</td>
<td>51.5</td>
<td>34.9</td>
<td>65.4</td>
<td>66.6</td>
<td>82.8</td>
<td>83.2</td>
</tr>
<tr>
<td>Leucotriene B₄ pg/ml</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
<td>&lt; detection limits</td>
</tr>
<tr>
<td>H₂O₂ μmol/l</td>
<td>0.28</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.46</td>
<td>0.12</td>
<td>n.a.</td>
</tr>
<tr>
<td>pH</td>
<td>7.9</td>
<td>8.1</td>
<td>8.1</td>
<td>8.4</td>
<td>8.5</td>
<td>8.2</td>
</tr>
</tbody>
</table>

n.a. not analysed
TABLE 4: Relation between body surface and box volume used for feral pigeons (*Columbia livia*) and domestic chickens to collect exhaled breath and exhaled breath condensates.

<table>
<thead>
<tr>
<th>unit</th>
<th>Pigeon</th>
<th>Chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average body surface*</td>
<td>cm²</td>
<td>553.8</td>
</tr>
<tr>
<td>Volume of box</td>
<td>l</td>
<td>15.6</td>
</tr>
<tr>
<td>Ratio box : bodysurface</td>
<td></td>
<td>1 : 35.5</td>
</tr>
</tbody>
</table>

* Average body surface = 10.0 x body mass (g)^{0.67} (Calder 1996)
FIG 1: Setup for the collection of exhaled breath (EB) and exhaled breath condensates (EBC) in an anaesthetized pigeon. A: 10 l respiratory bag filled with synthetic air (80% nitrogen and 20% oxygen); B: non-rebreathing valve, C: three-way switch, D: Collection of EB in a 6 ml vacutainer, E: ice-water-bath, F: collection tube for EBC.
FIG 2: Setup for the collection of exhaled breath and exhaled breath condensates in the chicken in an acrylic box. The inflow of air is at the top of the box, the outflow is at the bottom, with the tube led through a ice-water bath (~4°C).