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

J-M. Hatt, E. Zollinger, A. Boehler, M. Hofer, H. W. Steinmetz, M. Clauss

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]).

DISEASES of the respiratory tract are common causes of morbidity and mortality in birds (Lawton 1999, Tully 1995). Infections, especially mycoses caused by *Aspergillus* species, are frequently diagnosed postmortem, but it is difficult to diagnose respiratory tract disease in living birds, and a diagnosis is often made only when the disease has reached an advanced stage. Radiology, CT, and tracheopulmonic lavage are important diagnostic tools. However, in critically ill birds these methods may pose a risk because they often require general anaesthesia. Other diagnostic methods such as endoscopy of the trachea or coelomic cavity are invasive and also require general anaesthesia.

In recent years several improvements have been made in the treatment of avian diseases, especially of aspergillosis, but little progress has been made in the diagnosis of respiratory tract diseases. In human medicine the analysis of exhaled breath and exhaled breath condensate (EBC) plays an important role in the diagnosis of many lung diseases, including asthma, chronic obstructive pulmonary diseases (COPD), and bronchiectasis (Kharitonov and Barnes 2001). The method has the advantages, particularly for babies and children, that samples can be collected painlessly and frequently, and the response to any treatment can easily be assessed. The exhaled breath contains volatile compounds such as nitric oxide (NO) and carbon monoxide, and the EBC contains many biomarkers of oxidative stress and inflammation, including hydrogen peroxide (H2O2), leukotriene B4 (LTB4), prostaglandins and histamines. These mediators are released from the airway lining fluid into the exhaled breath and can be collected by condensing the exhalate in a cooled tube. Similar studies have been carried out with several species of mammals and reviewed by Zollinger and others (2006). It was concluded that for the method to become a useful diagnostic tool, further investigations are needed to evaluate the effect of variables such as sampling rate, sampling method, and sample storage temperature, and to develop a standardised collection protocol for each species.

No similar data have been published for birds, but in view of the prevalence of respiratory tract disease and the risk of stress, the analysis of exhaled breath and EBC appears to be a promising method. The aim of this study was to develop a method for collecting breath and EBC from feral pigeons (*Colombia livia*) and domestic chickens (*Gallus domesticus*), and to investigate whether inflammatory markers such as NO, H2O2, LTB4, and its pH could be measured.

Materials and methods

The study involved 20 feral pigeons and six domestic chickens, all of which were clinically healthy. The pigeons were adults and their bodyweight ranged from 340 to 450 g. The chickens were six months old and weighed between 2.5 to 3.3 kg. Before the study all the birds underwent a general health examination including haematology, blood chemistry, faecal parasitology and faecal bacteriology. Both species were kept...
in groups in outdoor aviaries. They were provided with a commercial pelleted diet and water ad libitum.

The study was evaluated and approved by the animal care and use committee of the Canton of Zurich (Permit 46/2005).

Two methods for collecting exhaled breath were investigated. In trial 1, 10 pigeons under general anaesthesia were intubated with a Cole’s endotracheal tube (Cook Veterinary Products), which was connected to a non-rebreathing valve (Model 2-W; Hugo Sachs Electronic-Harvard Apparatus) (Fig 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 filled with synthetic air (80 per cent nitrogen and 20 per cent oxygen). The expiratory tube was fitted with a three-way switch that allowed the separate collection of both breath and breath condensate. The breath was collected over an 18 G needle into a 6 ml vacutainer (Vacuette; Greiner Vacuette Diagnostik) to collect additional material.

FIG 1: Setup for the collection of exhaled breath (EB) and exhaled breath condensate (EBC) from an anaesthetised pigeon. A 10 l respiratory bag filled with synthetic air (80 per cent nitrogen and 20 per cent 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.

The pigeons in trial 1 were anaesthetised by the intramuscular administration of 0·2 mg/kg ketomedidine hydrochloride (Domitor; Dr E. Gräub), 100 mg/kg ketamine (Narketan 10%; Vétouqinol) and 0·2 mg/kg butorphanol (Morphasol; Vétouqinol). Repeated doses were given as necessary for a total period of anaesthesia of up to three hours. The effect of ketomedidine hydrochloride was reversed with atipamezole hydrochloride (Antisedan; Dr E. Gräub). While they were anaesthetised the pigeons were kept in a ventral position on a heated blanket, and their body temperature was measured continuously by a probe inserted into the crop (Cooper Instrument Corporation). Lactated Ringer’s solution was administered continuously through the ulnar vein at a rate of 3 ml/hour by an infusion pump (Injectomat; Fresenius).

The pH of the breath condensate was measured with a pH meter (691; Metrohm) within 20 minutes of collection and it was then frozen at −80°C until analysed for LTB4 and H2O2.

In trial 2, 10 pigeons and six chickens were maintained for up to two hours in an acrylic box (Fig 2). The measurements of the box for the pigeons were 0·25 × 0·25 × 0·25 m, and for the chickens the measurements were 0·5 × 0·5 × 0·5 m. The box was fixed tightly to the surface of the table with tape and was equipped with a thermometer and a hygrometer. The access for synthetic air was through an inlet in the top of the box and there was an outlet near the floor. The flow rate of synthetic air for the pigeons was 0·9 l/minute and for the chickens if was 6·6 l/minute.

The samples were collected in the same way as in trial 1, beginning 90 minutes after the bird had been placed in the box.

Samples for the analysis of NO were divided into two groups. One set was stored on ice until it was analysed within one hour of collection. The other set was kept at ambient temperature and the NO concentration was measured with a chemiluminescence analyser (CLB 88 sp; Eco medics) with a range of detection between 0·1 and 5000 ppb.

Before they were analysed for H2O2, the condensate samples were allowed to reach ambient temperature; 300 µl of the condensate was then mixed with an equal volume of phosphate buffer and H2O2 was measured with a biosensor (EcoCheck H2O2; FILP Lungen- und Thorax-diagnostik). According to the manufacturer the limit of detection was 15 nmol/l. A commercial enzyme immunoassay was used to measure LTB4 (Leukotriene B4 ELISA kit; Cayman Chemical Company), according to the manufacturer’s instructions.

The data were analysed by using a software package for personal computers (Statistika 7.1; StatSoft). Statistical significance was set at P<0·05. The objective of the analyses was to determine whether there was a difference between the measurements made in the two trials and whether there was a relationship between the measurements and the sampling and storage methods. The results are given as mean (sd).

The effects of the storage methods and the two species of birds were analysed by the paired t test, and the influence of the sampling methods was analysed by the two-sample test. Whether the data from each type of sample were normally distributed and had the same variance were tested by the Kolmogorov-Smirnov test and Hartley Fmax-test, respectively. If they were not, or the sample number was below five, the alternative non-parametric Mann-Whitney U test or Wilcoxon matched pairs test were used. The Pearson product-moment correlation coefficient was calculated for the relationships between the measurements of breath condensate and the birds’ bodyweight.

Results

All the birds appeared clinically healthy throughout the study, but pigeon 7 died while anaesthetised. A postmortem investigation revealed a fresh haemorrhage in its coelomic cavity, but there were no external signs of trauma and the aetiology for the haemorrhage could not be determined.

The samples of condensate stored on ice had significantly higher concentrations of NO (99·6 [37·0] ppb) than the samples stored at ambient temperature (80·7 [33·4] ppb) (P<0·001).

In trial 1, between 0·17 and 0·46 ml of condensate was collected within three hours (Table 1). In pigeons 1 to 4 the volume of condensate was not measured and 0·20 to 0·35 ml were used for the analysis. For the comparison of the volumes of condensate in this species these ranges...
Below the detection limit in all the samples, and the concentration of LTB4 was below the detection level in all the birds.

In contrast with trial 1 (7.9 [0.3] v 5.3 [0.1], P<0.001) and the chickens had significantly higher pH values than the pigeons (8.2 [0.2] v 7.9 [0.3], P=0.04).

The mean volume of condensate obtained from pigeons in the acrylic box (1.66 [0.64] ml/kg) was significantly greater (P<0.001) than from the intubated pigeons (0.15 [0.06] ml/kg) was significantly smaller (P<0.001) than from the chickens. The mean volume of condensate collected from the pigeons in the acrylic box was approximately twice as large for the chickens as for the pigeons (Table 4).

The mean volume of condensate obtained from pigeons in the acrylic box (1.66 [0.64] ml/kg) was significantly greater (P<0.001) than from the intubated pigeons (0.87 [0.32] ml/kg). However, the volumes obtained from the six intubated pigeons (0.40 to 1.26 ml/kg) and the 10 pigeons maintained in an acrylic box (1.03 to 2.92 ml/kg) were independent of their bodyweight. The mean volume of condensate collected from the chickens (0.15 [0.06] ml/kg) was significantly smaller (P<0.001) than from the pigeons (1.66 [0.64] ml/kg). During the three hours spent by the pigeons in the acrylic box there were significant changes in temperature (P=0.04) and relative humidity (P=0.01), but no significant changes were observed with the chickens. The concentrations of NO in the uncooled samples of breath from the chickens and pigeons kept in the box were lower than the concentrations in cooled samples but, in contrast with trial 1, the difference was not significant (cooled 72.7 [24.5] ppb v 89 [19.7] ppb; uncooled 64.1 [18.6] ppb v 70 [13.3] ppb). In contrast with trial 1, H2O2 could be measured in all but one pigeon. In contrast with trial 1, H2O2 could be measured in all but one pigeon.

In the present study, cooled (4°C) samples resulted in higher NO values in pigeons this difference was significant. This is in contrast to Bodini and others (2003), who found that storage of NO samples for two-and-a-half times more condensate was collected from the pigeons when they were kept in an acrylic box than when they were intubated. This method appears to be less stressful and particularly useful for sick birds. However, it carries certain risks of sample contamination from excretions and cutaneous water loss, as described in pigeons by Webster and King (1987), and led to a significant increase in relative humidity. Differences in cutaneous water loss may explain why the pigeons produced larger volumes of condensate than the chickens. Alternatively, the difference in EBC volumes between pigeons and chickens could also be explained by the fact that chickens had a larger box than the pigeons compared to their body surface (Table 4) and a higher air flow.

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 1999, 2000). 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. Also pigeons produced approximately four times less EBC volume per kg body mass compared to cats, dogs, calves and horses (Reinhold and others 1999, 2000, Deaton and others 2001, Pietra and others 2003, Sparkes and others 2004, Wyse and others 2004).

In the present study, the parameters to be analysed (H2O2, 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 on different mammalian species, mostly on clinically healthy animals. However, studies have been conducted on calves and horses with disease. In calves, intra-tracheal inoculation with Pasteurella multocida resulted in a significant elevation of LTB4 in the EBC within three days (Reinhold and others 1999, 2000). 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 H2O2 in the EBC was significantly increased (Deaton and others 2002).

In the present study, cooled (4°C) samples resulted in higher NO values in pigeons this difference was significant. This is in contrast to Bodini and others (2003), who found that storage of NO samples for 9 hours at 4°C, 21°C or 37°C had no effect on concentration. NO sam-

### Table 1: Analysis of exhaled breath (nitrous oxide [NO]) and exhaled condensate (H2O2, pH and leukotriene B4) in intubated clinically healthy feral pigeons (Columba livia) under general injectable anaesthesia

<table>
<thead>
<tr>
<th>Pigeon</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>339</td>
<td>380</td>
<td>340</td>
<td>420</td>
<td>364</td>
<td>394</td>
<td>420</td>
<td>392</td>
<td>420</td>
<td>390</td>
</tr>
<tr>
<td>Exhaled breath condensate (ml)</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>0.46</td>
<td>0.30</td>
<td>0.17</td>
<td>0.40</td>
<td>0.46</td>
</tr>
<tr>
<td>NO (ppb)*</td>
<td>88.0</td>
<td>105.0</td>
<td>86.4</td>
<td>61.1</td>
<td>207.7</td>
<td>123.7</td>
<td>120.4</td>
<td>108.4</td>
<td>183.4</td>
<td>136.9</td>
</tr>
<tr>
<td>Uncooled</td>
<td>71.6</td>
<td>54.2</td>
<td>85.2</td>
<td>32.2</td>
<td>174.0</td>
<td>112.3</td>
<td>95.5</td>
<td>95.7</td>
<td>155.2</td>
<td>126.5</td>
</tr>
<tr>
<td>Leukotriene B4 (pg/ml)</td>
<td>7.3</td>
<td>5.3</td>
<td>25.7</td>
<td>5.0</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
</tr>
<tr>
<td>H2O2 (µmol/l)</td>
<td>5.6</td>
<td>4.3</td>
<td>5.3</td>
<td>5.3</td>
<td>5.26</td>
<td>5.3</td>
<td>5.2</td>
<td>5.3</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>2.46</td>
<td>2.46</td>
<td>2.46</td>
<td>2.46</td>
<td>2.46</td>
<td>2.46</td>
</tr>
</tbody>
</table>

* Cooled NO samples were stored on ice until analysis, uncooled samples were stored at room temperature.

### Table 2: Analysis of exhaled breath (nitrous oxide [NO]) and exhaled condensate (H2O2, pH and leukotriene B4) in clinically healthy feral pigeons (Columba livia) maintained in an acrylic box

<table>
<thead>
<tr>
<th>Pigeon</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>426</td>
<td>338</td>
<td>390</td>
<td>390</td>
<td>390</td>
<td>390</td>
<td>424</td>
<td>386</td>
<td>446</td>
<td>432</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>
<td>0.76</td>
<td>0.79</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Temperature in box (start/endpoint) (°C)</td>
<td>NA</td>
<td>NA</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>
<td>22/22</td>
</tr>
<tr>
<td>Relative humidity (start/endpoint) (%)</td>
<td>NA</td>
<td>NA</td>
<td>45/76</td>
<td>45/60</td>
<td>45/60</td>
<td>45/55</td>
<td>45/55</td>
<td>45/55</td>
<td>45/55</td>
<td>45/55</td>
</tr>
<tr>
<td>NO (ppb)*</td>
<td>95.5</td>
<td>78.7</td>
<td>NA</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>
<td>104.9</td>
</tr>
<tr>
<td>Uncooled</td>
<td>70.1</td>
<td>61.2</td>
<td>NA</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>
<td>72.2</td>
</tr>
<tr>
<td>Leukotriene B4 (pg/ml)</td>
<td>0.16</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
<td>UDL</td>
</tr>
<tr>
<td>H2O2 (µmol/l)</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>
<td>8.1</td>
</tr>
</tbody>
</table>

* Cooled NO samples were stored on ice until analysis, uncooled samples were stored at room temperature.

NA Not analysed, UDL Under detection limit.
For the analysis of H₂O₂, it was found that the necessary sample volume of 300 μl was prohibitively large, and allowed the measurement of H₂O₂ only in the animals in the box. Diluting the sample by adding 250 μl of aqua bidestillata to a 50 μl aliquot was tried, 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 mammals (Zollinger and others 2006). The only exception was pigeon 3, which had a 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 indicating that the elevated first value was likely to have been caused by an analytical error. 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 is possible that concentrations decreased due to degradation processes, this could explain why none of the birds sampled in the box had detectable LTB₄ values, as these samples were analysed after a longer period than samples from trial 1. In future it should be recommended to freeze samples, which could be analysed immediately after thawing.

The pH of EBC in the pigeons in the box was 2 to 3 log higher than in the intubated pigeons.

Exhaled breath and EBC can successfully be collected and parameters determined in chickens and pigeons, which in mammals are considered indicators for inflammation. However, the interpretation of the results and comparison with other studies is challenging due to the wide range in the concentration of inflammatory markers and the different methods used. For further development of exhaled breath and EBC analysis it is therefore important that the sample collection procedure is standardized.

Further systematic studies with additional species, such as psittacine birds or raptors, and birds with respiratory tract pathology are needed to determine the diagnostic application of exhaled breath and EBC analysis. The technical equipment described in the present study is inexpensive and easy to install. If pH could be detected as a diagnostic parameter this would be useful since analysis could be easily performed in a clinical setting. The other parameters NO, LTB₄, and H₂O₂ might prove to be less useful in practice, as analysis would need to be carried out in a specialised laboratory. However, future development of online NO measurements might produce affordable online measurement equipment for veterinary practice.

Acknowledgements

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