UNIVERSITY OF CALIFORNIA,
IRVINE

Investigation into the Utility of Gas Signature Detection as a Diagnostic Tool for Infectious Diseases

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Charlotte Marie Hirsch

Dissertation Committee:
Professor Donald Blake, Chair
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2016

DEDICATION

To

My loving family, including our little one arriving this August

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ABSTRACT OF THE DISSERTATION

Investigation into the Utility of Gas Signature Detection as a Diagnostic Tool for Infectious Diseases

By

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Doctor of Philosophy in Chemistry

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Professor Donald Blake, Chair

This thesis will explore the diagnostic and monitoring potential of exhaled breath for various infectious diseases. The specific infections studied include *Borrelia hermsii, salmonella enterica*, and two forms of endotoxin. Samples of breath were collected from mice both individually and in groups as well as from rats. The animals were awake and restrained in a breath sample tower for a majority of the studies. One study was also conducted using awake unrestrained rats in a glass bulb that was designed for breath sample collection by the Rowland Blake lab. In addition to the breath samples, feces samples were obtained from mice infected with *salmonella enterica* and incubated in bioreactors from which the headspace was collected for analysis. Samples were collected in evacuated stainless steel canisters. Blank samples were also collected to determine concentrations in the air used during sampling.

All samples, excluding the feces samples, were analyzed for carbon monoxide and carbon dioxide concentrations using gas chromatography. Several samples were also analyzed for various volatile organic compounds using the Rowland Blake non-methane

hydrocarbon gas chromatography system. The results of the gas chromatography measurements were analyzed to determine if differences existed between infected and uninfected samples throughout each study. This was accomplished statistically, as well as visually using plots.

The results of the *Borrelia* and endotoxin studies revealed a significant increase in the ratio of CO/CO_2 in the breath of the infected mice and rats, with a dose response being observed for the endotoxin study. Upon treatment, the ratio of CO/CO_2 returned to normal within several hours of antibiotic administration. For the *salmonella* study, carbonyl sulfide, dimethyl disulfide, carbon disulfide, heptanal, i-propylbenzene, and isoprene were identified in the feces; and acetone, isopropanol, and a gas of unknown identity in the breath. The endotoxin study identified i-pentane in the breath of mice. The study with rats identified two gases, acetone and an unknown gas. The results of these studies suggest that exhaled breath has potential for monitoring of the infections studied, however further studies are needed to explore the diagnostic potential.

CHAPTER 1: Introduction

Infectious diseases are those illnesses which are caused by a contagious pathogen, such as a bacterium, fungus, virus, or parasite [1, 2]. While advancements in hygiene practices and medicine over the past few decades have decreased the occurrence and mortality of many infectious diseases, these diseases still contribute significantly to the global health burden [2]. According to the Centers for Disease Control and Prevention (CDC), in 2010 the number of visits to physician offices for infectious disease related illnesses was 23.6 million in the United States alone [3]. More specifically in 2012 there were 30,800 new cases of Lyme disease and 53,800 new cases of salmonella [4].

The treatments and outcomes for infectious diseases vary based on the pathogen(s) involved. In the case of bacterial infections for example, the treatment requires the use of antibiotics. One major problem with this is the issue of antibiotic resistance among the bacteria [5]. Because of difficulty in diagnosing these diseases, antibiotics tend to be overprescribed leading to overuse or misuse, which can eventually lead to the bacteria becoming resistant to the drug. Antibiotic resistance is a global issue, increasing in all parts of the world and leading to more lengthy and costly medical care [5, 6]. It is estimated that antibiotic resistant bacteria related healthcare costs and productivity losses in the European Union total over US $1.5 billion per year and account for 25,000 deaths [7].

While new diagnostic tools and techniques are continuously being developed, several challenges remain unmet [8]. There is an ever growing need for diagnostic tools that are specific to certain pathogens, i.e. bacterial, viral, or fungal, for example to limit over prescribing of antibiotics [9]. Additionally, the time needed for detection in current diagnostic techniques varies from less than a minute in the case of microscopy, to weeks in

the case of cultures [9]. For these reasons, diagnostic tools for infectious diseases that are non-invasive, rapid and specific are needed now more than ever.

When an infectious disease occurs, the pathogen generally enters the blood stream where it can spread throughout the body [10]. For this reason, many of the current diagnostic techniques used focus on the collection of samples of blood. In addition to the above mentioned issue of time, the use of blood samples for diagnosis requires resources and potentially endangers both the patient and the person performing the blood sample due to the use of needles and exposure to contaminated blood [11]. For this reason, a technique that can detect a disease agent that is present in a patient's blood without the use of a blood sample would be preferred.

In the human body, the main goal of the lungs is to exchange carbon dioxide (CO_2) excreted from tissues, with oxygen (O_2) from the air [12]. This is accomplished primarily via capillaries, which are tiny blood vessels connecting the lungs with all the organs of the body (Figure 1.1a) [12, 13]. On one end of the chain, the capillaries are connected to the pulmonary artery which brings deoxygenated blood, containing CO_2, from other tissues and organs in the body [13]. Here gas exchange is performed via sacs of lung tissue called alveoli which allow dissolved CO_2 out of the blood and gaseous O_2 in (Figure 1.1b) [12]. The pulmonary vein then returns the newly oxygenated blood to the organs and tissues in the rest of the body [12].

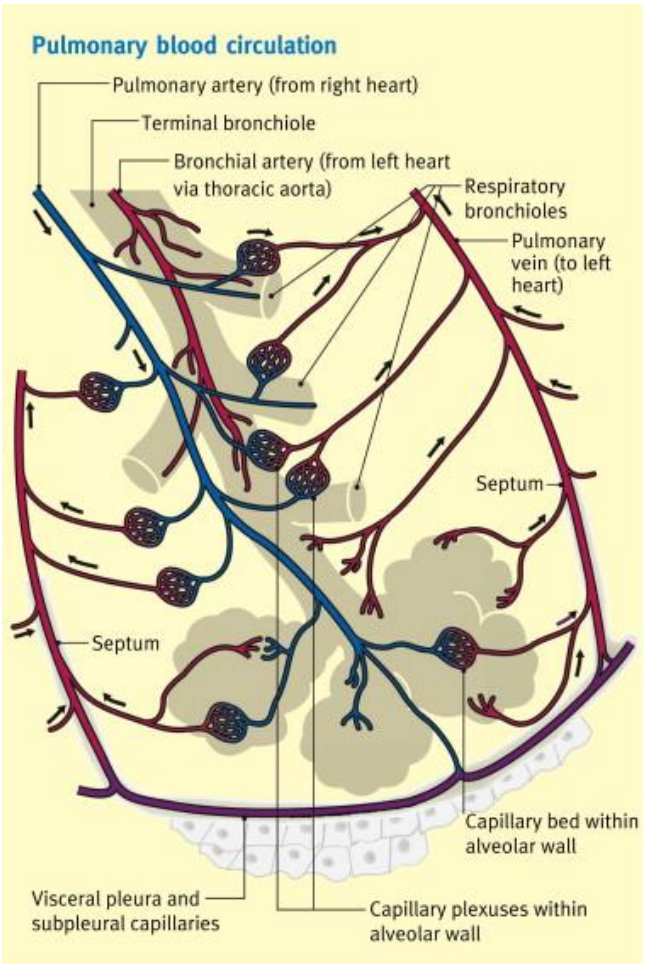

Figure 1.1a. Schematic of capillary network in lungs [13].

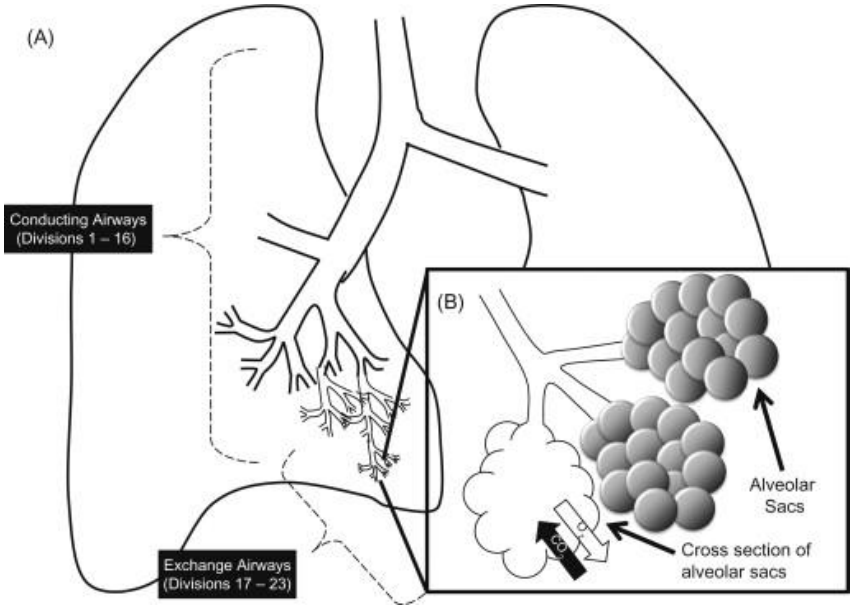

Figure 1.1b. Schematic of main gas exchange in lungs occurring at alveoli [12].

Because of the gas exchange known to occur between the blood stream and the lungs, it is likely that the gases in exhaled breath could be representative of those in the blood stream. Therefore if a pathogen in the blood stream causes differences in the blood gas concentrations, this change should be reflected in the gases of the patient's breath. Recently in fact, the use of breath gas signatures as a tool for diagnosing and monitoring diseases has been gaining traction. The idea behind using bodily odor, including breath, to aid in diagnosing diseases however is all but recent [14].

In fact since ancient times doctors have been noting the fact that certain diseases carry with them noticeable odors [15]. For example, a diabetic who is experiencing ketoacidosis may have a fruity or sweet breath smell [15]. Ketoacidosis results from the body breaking down fat into ketones which can exit the body through the lungs [16]. Acetone, a ketone which could be described as having a fruity or sweet odor, has in fact been detected in exhaled breath of patients with diabetes mellitus [17]. Another disease with an associated bodily odor is maple syrup urine disease in which a buildup of amino acids in the body leads to a sweet scent in the urine [18].

While these odors can be readily detected by the human nose, there may be numerous other diseases and conditions which cause changes in the gas signature of the patients' breath and body that may not be possible to detect in this manner. Further evidence for this exists in the fact that canines are able to detect a number of medical conditions by detecting changes in the composite odors of humans [19]. A study published in 2014 found that two canines were able to detect prostate cancer in the urine of patients affected with the disease with 98.6-100% sensitivity and 97.6-98.7% specificity [20]. Additionally, since the 1990s dogs have been trained for use as seizure alert dogs for

medical assistance in patients with serious seizure disorders [21]. These dogs are able to alert the patient to an oncoming seizure that not even the patient is aware of [19, 21]. Today diagnostic, response and alert dogs are not only used in patients with seizure disorders but also in a multitude of other diseases from Parkinson's to diabetes [19].

It has been suggested that the dogs in these cases are detecting a change in odor of the patients caused by the respective condition [19, 21]. This remarkable ability is a result of the higher sensitivity of the canine nose when compared to humans. The chemicals responsible for the odor the canines detect are classified as volatile organic compounds, or VOCs. In the case of certain VOCs for example, the canine nose can be over 100 times more sensitive than those of humans [22, 23]. This is likely caused by the anatomical differences in the olfactory systems of humans and canines [23, 24]. The olfactory systems of canines and humans have been extensively studied and it has been determined that those of the canines are anatomically more complex. In canines for example, olfactory epithelium accounts for 45-50% of the nasal epithelium compared to only 5% in humans [24]. A summary of some of the major anatomical differences between the olfactory systems of humans and canines is shows in Table 1.1 [24].

Table 1.1. Anatomical Differences in Canine and Human Olfactory Systems [24].

Anatomical Structure	Human	Canine (Beagle)
Vestibule	Relatively large	Relatively small
Ethmoturbinates	Simple unbranched	Complex branched
Vomeronasal organ	Absent in adults	Present
Nasopalatine ducts	Absent in adults	Present
Steno's glands	Absent	Present
Nasopharyngeal septum	Absent	Present
Transverse lamina and olfactory recess	Absent	Present

In order to be able to use human breath and body odors to diagnose diseases, a more sensitive instrument than the human nose is needed. Additionally, in order to use the changes in breath gas signatures throughout the course of various diseases to determine the presence of a specific marker or markers for those diseases, the ability to detect very low concentrations of gases is of great importance. One technique that can be used to measure concentrations of gases with this much needed high sensitivity is gas chromatography.

Gas chromatography involves the separation of a mixture of gases into its individual component gases [25]. This analytical technique has the ability to detect a multitude of gases including hydrocarbons, halocarbons, and sulfur compounds among others [25]. The Rowland-Blake laboratory at the University of California Irvine has performed atmospheric monitoring for decades using a highly tuned multicolumn/detector gas chromatography system capable of detecting gases from the parts per million (ppm) to the parts per quadrillion (ppq) range [26]. Recently, an entire analytical system has been dedicated to breath gas measurement which is identical to the atmospheric system. A list of the gases quantified by the Rowland Blake laboratory is shown in Table 1.2 below. In addition to the listed gases, CO and CO_2 can be measured using a separate system.

Table 1.2. a. List of gases measured using gas chromatography in Rowland Blake laboratory at University of California Irvine.

Hydrocarbons	Formula	Hydrocarbons	Formula	Hydrocarbons	Formula
Methane	CH_4	*i*-Pentane	C_5H_{12}	*i*-Propylbenzene	C_9H_{12}
Ethane	C_2H_6	*n*-Pentane	C_5H_{12}	*n*-Propylbenzene	C_9H_{12}
Ethene	C_2H_4	Isoprene	C_5H_8	4-Ethyltoluene	C_9H_{12}
Ethyne	C_2H_2	*n*-Hexane	C_6H_{14}	3-Ethyltoluene	C_9H_{12}
Propane	C_3H_8	*n*-Heptane	C_7H_{16}	2-Ethyltoluene	C_9H_{12}
Propene	C_3H_6	*n*-Octane	C_8H_{18}	1,2,4-Trimethylbenzene	C_9H_{12}
i-Butane	C_4H_{10}	Benzene	C_6H_6	1,3,5-Trimethylbenzene	C_9H_{12}
n-Butane	C_4H_{10}	Toluene	C_7H_8	1,2,3-Trimethylbenzene	C_9H_{12}
cis-2-Butene	C_4H_8	Ethylbenzene	C_8H_{10}	alpha-Pinene	$C_{10}H_{16}$
trans-2-Butene	C_4H_8	*m/p*-Xylene	C_8H_{10}	beta-Pinene	$C_{10}H_{16}$
1-Butene/*i*-Butene	C_4H_8	*o*-Xylene	C_8H_{10}	Limonene	$C_{10}H_{16}$

Table 1.2. b. List of gases measured using gas chromatography in Rowland Blake laboratory at University of California Irvine continued.

Oxygenates	Formula	Halocarbons	Formula
Methanol	CH_3OH	Trichloromethane	$CHCl_3$
Ethanol	CH_3CH_2OH	1,1,1-Trichloroethane	CH_3CCl_3
Isopropanol	C_3H_8O	Tetrachloromethane	CCl_4
Butanal	C_4H_8O	Dichloromethane	CH_2Cl_2
Acetaldehyde	CH_3CHO	Trichloroethene	C_2HCl_3
Acetone	CH_3COCH_3	Tetrachloroethene	C_2Cl_4
Butanone	C_4H_8O	Methyl chloride	CH_3Cl
2- & 3-Pentanone	$C_5H_{10}O$	Ethyl chloride	CH_3CH_2Cl
Alkyl Nitrates	**Formula**	Methyl iodide	CH_3I
Methyl nitrate	CH_3ONO_2	Methyl bromide	CH_3Br
Ethyl nitrate	$C_2H_5ONO_2$	Dibromomethane	CH_2Br_2
n-Propyl nitrate	$C_3H_7ONO_2$	Tribromomethane	$CHBr_3$
i-Propyl nitrate	$C_3H_7ONO_2$	Bromodichloromethane	$CHBrCl_2$
2-Butyl nitrate	$C_4H_9ONO_2$	CFC-11	$CFCl_3$
2-Pentyl nitrate	$C_5H_{11}ONO_2$	CFC-12	CF_2Cl_2
3-Pentyl nitrate	$C_5H_{11}ONO_2$	CFC-113	CCl_2FCClF_2
3-Methyl-2-butyl nitrate	$C_5H_{11}ONO_2$	CFC-114	$C_2Cl_2F_4$
Sulfur Compounds	**Formula**	H-1211	$CBrClF_2$
Carbonyl sulfide	OCS	HCFC-141b	CH_3CCl_2F
Carbon disulfide	CS_2	HCFC-142b	CH_2CCl_2F
Dimethyl sulfide	CH_3SCH_3	HCFC-22	CHF_2Cl
Dimethyl disulfide	CH_3SSCH_3	HFC-134a	CH_2FCF_3
Dimethyl trisulfide	CH_3SSSCH_3	HFC-152a	$C_2H_4F_2$

The use of breath as a representation of blood gas concentration was first used in a hand held electronic device in the mid- 1970s [27]. This device was capable of detecting alcohol concentration in the breath and by means of a calculation using a blood to breath ratio calibration factor, the blood alcohol concentration can be determined [27, 28]. It has also been determined that certain processes in the body result in the production of VOCs which make their way through the blood stream to the lungs where they will be excreted during the gas exchange in the alveoli [29, 30]. For example, lipid peroxidation in the body can lead to the production of acetone which is one of the major components of human breath [30]. Additionally, isoprene is produced in the body during cholesterol synthesis and has also been detected in human breath [17]. A summary of some gases found in human breath and their possible sources is shown in Table 1.3 [17, 31].

Table 1.3. List of some gases in human breath and potential sources in the body [Adapted from 17 and 31].

Name	Formula	Potential source
Acetone	C_3H_6O	Lipolysis
Carbon dioxide	CO_2	Oxidative by product of energy substrates
Carbon monoxide	CO	Marker of oxidative stress
Ethane	C_2H_6	Lipid peroxidation
Ethanol	C_2H_6O	Fermentation of sugars in intestine
Isoprene	C_5H_8	Cholesterol synthesis
Hexanal	$C_6H_{12}O$	Natural waste product
Methyl nitrate	CH_3NO_3	Peroxy radical interaction with NO
Pentane	C_5H_{12}	Lipid peroxidation
Propane	C_3H_8	Protein oxidation

Summary

The ability to estimate blood gas concentrations in a non-invasive manner makes the use of exhaled breath gas signatures as a diagnostic tool for infectious diseases quite promising [32, 33]. In this dissertation, the changes in breath gas signatures throughout

the course of infections with various diseases will be examined to elucidate possible markers that could be used for diagnosis and monitoring of the diseases. To determine the feasibility of this technique, animal models will first be used. In addition to breath, the gases collected from the headspace of feces samples of infected and control animals will also be discussed for comparison to the breath samples. This would allow for a possible determination of whether the gases observed are coming from the bacteria in the gut or as a result of the infection in the body. The studies discussed in this thesis are largely pilot studies involving small sample sizes and short sample periods to determine the validity of exhaled breath samples for use in diagnosis of the pathogens investigated.

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CHAPTER 2: Methods

Summary of studies

This dissertation will discuss studies involving the following 3 pathogens, *Borrelia Hermsii, Salmonella enterica,* and lipopolysaccharide (LPS) endotoxin from *Esceria coli*. These studies were performed in collaboration with the labs of Dr. Manuela Raffatellu in the case of *Salmonella* and Dr. Alan Barbour for all other studies. For the *Borrelia* infections, 4 separate studies were performed, using two different types of mice. The first type sampled was the normal Balb-C strain, which is an albino strain of the common house mouse, while the second type was a severe combined immunodeficiency (SCID) mutated Balb-C strain [1]. Each strain of mouse was sampled in two separate studies. Mice were acquired, trained, handled and infected by Dr. Barbour's lab. The sampling strategies for each study will be discussed in greater detail in subsequent chapters, but all involved the collection and analysis of breath samples from uninfected and infected mice for comparison.

The *Salmonella* studies included a breath study conducted using groups of mice in a breath collection tower in which breath samples were collected from healthy and infected mice for comparison. In addition, a study of the headspace of feces of infected and control mice was performed using bioreactors. Finally, there were four LPS endotoxin studies conducted using the exhalation tower, three of which were mouse studies and one that used rats. Additionally, in the LPS rat study breath samples were collected from the rats using sealed glass bulbs that had been designed for this purpose for comparison to the tower. Table 2.1 summarizes the studies performed and the manner in which samples were collected as $breath_{tower}$ (Tower), $breath_{bulb}$ (Bulb), or headspace (HS).

Table 2.1. Summary of projects discussed in this dissertation.

Study name	Collection type	# of samples
Salmonella feces	HS	20
Salmonella mice	Tower	35
Borrelia Balb-C mice 1	Tower	63
Borrelia Balb-C mice 2	Tower	20
Borrelia SCID mice 1	Tower	180
Borrelia SCID mice 2	Tower	188
Endotoxin Mice 1	Tower	97
Endotoxin mice 2	Tower	45
Endotoxin mice 3	Tower	33
Endotoxin mice 4	Tower	21
Endotoxin Rats a	Tower	50
Endotoxin Rats b	Bulb	24
Total samples		**776**

Stainless steel canister preparation

All air samples discussed in this dissertation, breath, blank, or head-space, were collected in 1.9 L stainless steel canisters. Prior to evacuation for use, the canisters used for breath and headspace samples were baked in a humidified oven (Fisher Scientific, Houston, TX) at 150°C for twelve hours [2]. After the baking process, these canisters were treated in the same manner as all other samples. Canisters were then connected in groups of up to eight, to a pump-out line using Swagelok ultra-torr connectors and stainless steel flex tubing where they were evacuated. They were then filled to 500 torr, with 99.999% pure helium (UHP) which had been further purified using a cryogenically cooled stainless steel trap containing molecular sieves 5A and 13x as well as activated charcoal [2]. Finally, the canisters were again evacuated to a pressure of about 1×10^{-2} torr. The system used for the pump out process is pictured in Figure 2.1.

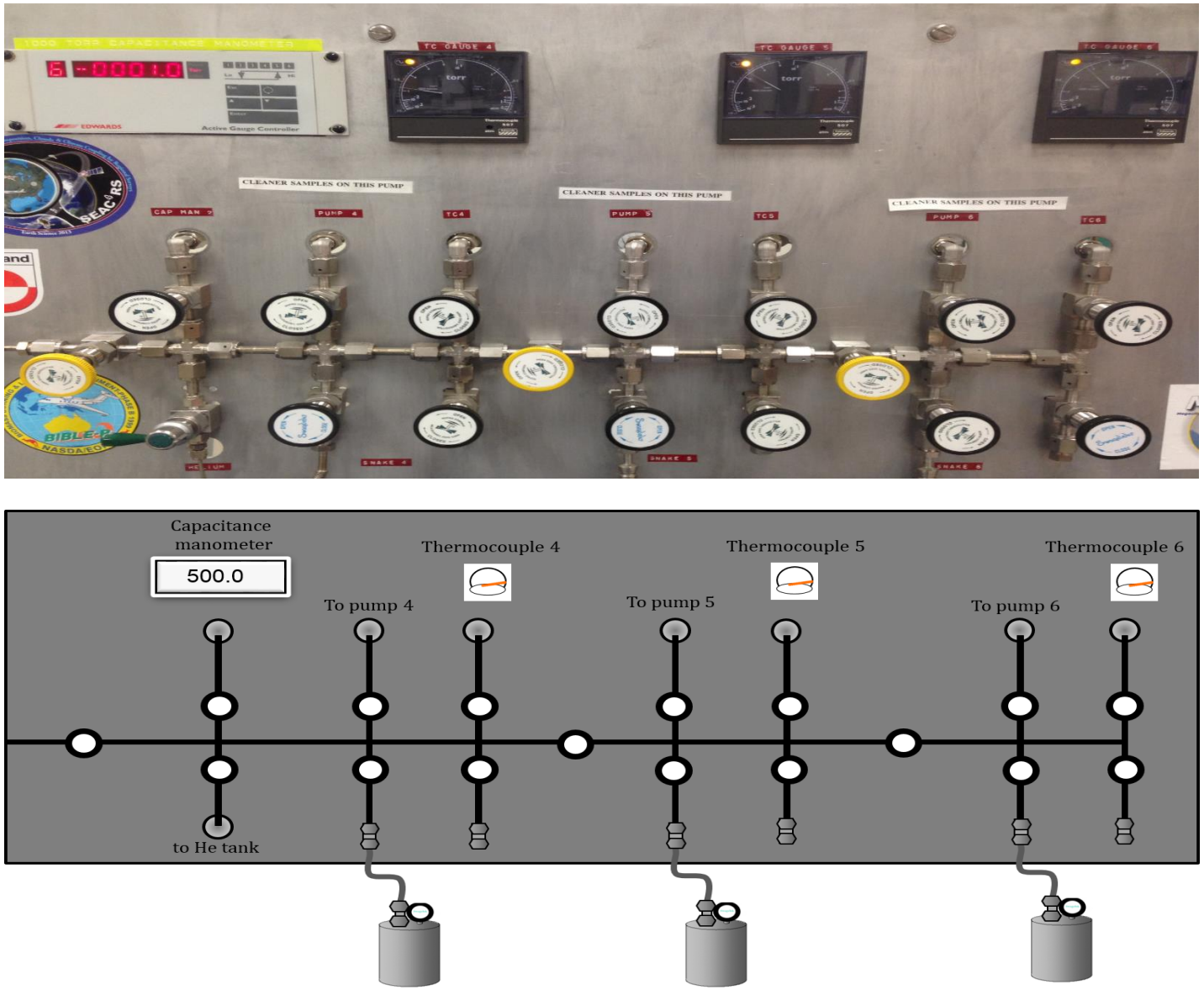

Figure 2.1. Picture and schematic of manifold used to evacuate canisters during preparation (3 of 6 pump valve lines shown).

Exhaled breath sample collection from mice or rats using tower apparatus

Breath samples were collected from awake mice and rats using a nose-only cylindrical inhalation tower provided by the Barbour laboratory. This apparatus is an exposure tower obtained from In-Tox Products (Moriarty, New Mexico), that originally was designed for the introduction of aerosols to mice, either in groups or individually. The tower contains a straight tube connecting to twelve ports, at which mice can be "exposed" via O-ring sealed flow-by nose pieces with 28 mm outlets. Each of the twelve ports can be sealed off to allow for anywhere from one to twelve mice to be attached at one time. The mice were placed in Lexan restraint tubes measuring 9.0 cm long with an inner diameter of 3.1 cm which attach to the tower via the O-ring sealed nose ports. The restraint tubes used for the rats measure 19.0 cm in length with an inner diameter of 5.7 cm. The rat tubes also connect to the tower via O-ring sealed nose-only ports. The two restraint tubes were obtained from In-Tox products and are pictured in Figures 2.2a and b.

Figure 2.2a. Rat restraint tube with nose only port attached (top) compared to mouse restraint tube with nose only port attached (bottom).

Figure 2.2b. Nose only port side view (left), interior view (middle), front view with rat attached (right)

The exposure tower was redesigned as a breath collection tower and used as follows. The inlet and outlet of the inhalation tower were fitted with Swagelok ultra-torr and stainless steel flex tubing connections to allow for the introduction of ultra-pure air from a pressurized cylinder. The air from the cylinder was collected from the University of California White Mountain Research Station at approximately 3,100 meters elevation in the Sierra Nevada Mountains to ensure clean background concentrations of gases. The air flow was directed into the tower through the central tube to the un-sealed ports, where it would pass through the nose port and into the animal. The animal then exhaled the air which was directed back through the outlet of the tower, with the continuously flowing White Mountain air, where it exited through the attached HEPA filter. The filter outlet was connected via ultra-torr connectors and flex tubing to an evacuated stainless steel canister where the mixed air was collected and stored for analysis. An absolute pressure gauge was attached at the base of the tower to allow for continuous monitoring of pressure on the animals. Figure 2.3 is a photograph showing the collection tower with mouse in restraint tube attached.

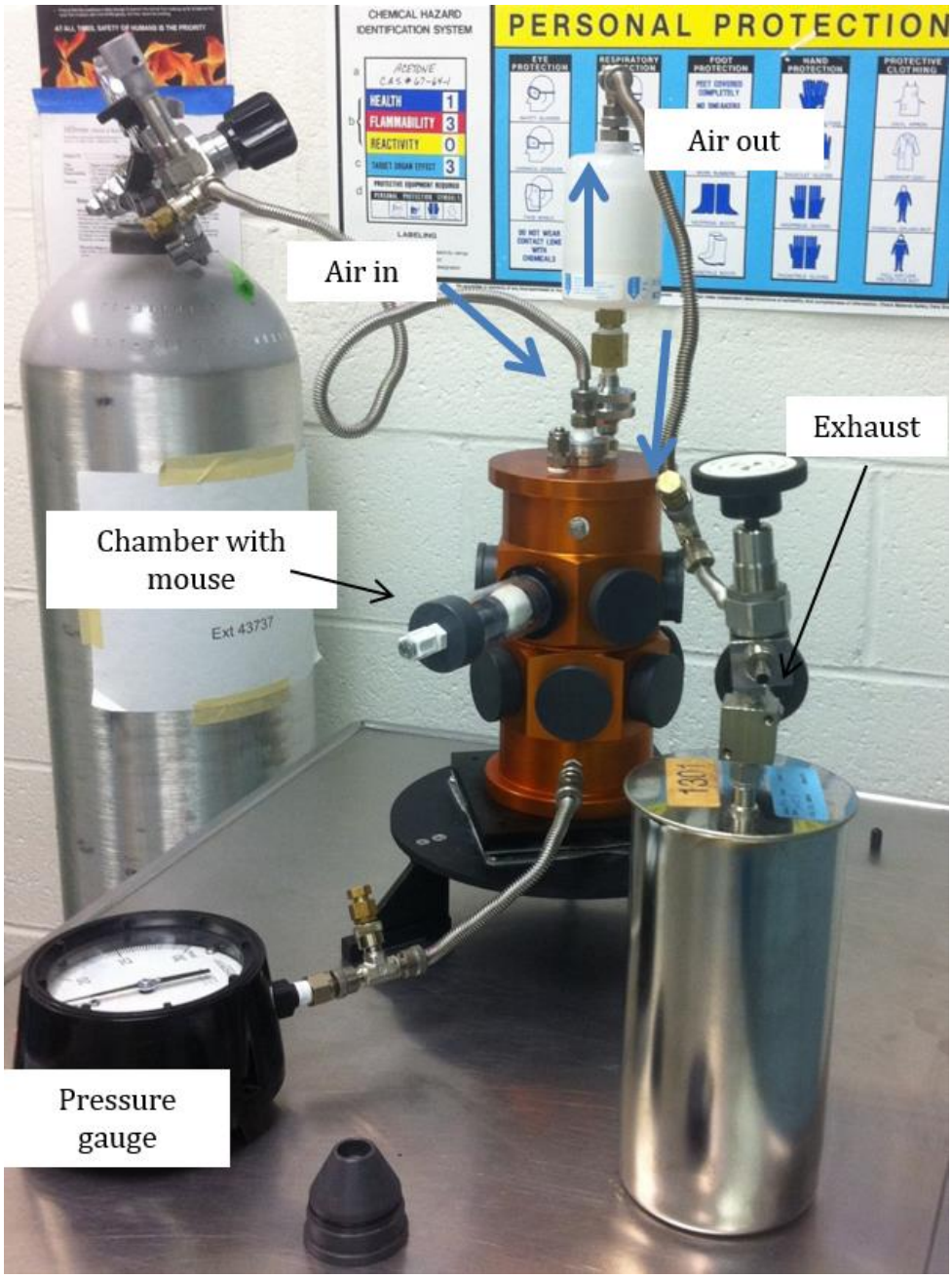

Figure 2.3. Breath sample tower set-up for collection from individual mice.

One challenge with the use of the tower for breath collection was that because of the constant flow of air through the tower during sampling, there was a dilution of the exhaled breath sample as it mixed with this background air. In the initial studies, a flow rate of 1 L/min was flushed through the tower for six minutes after which the exhaust valve was

closed and the evacuated canister was opened to collect the air from the tower for approximately two minutes. This high flow rate was used in order to decrease the amount of stress on the animals while they were contained in the tower. An attempt was made to increase the breath to background concentration in the canisters for individual mice by decreasing the flow rate without further stress on the mice, in a small study.

The results of this study indicated the sampling could be optimized as follows; the flush rate remained at 1 L/min for six minutes after which the flow was reduced to 0.5 L/min for an additional minute before the exhaust valve was closed and the canister was opened to collect the air from the tower for approximately four minutes. The decreased sample flow rate allowed for the breath sample to be diluted less, while keeping the flush rate higher reduced the amount of time the animal was restrained in the tower. For all experiments, canisters were also collected from an empty restraint tube or tubes connected to the tower to serve as blank samples, prior to collection of animal breath samples, at the same flow rates. A summary of the breath studies conducted using the tower and the sample flow rates for each is shown in Table 2.2.

Table 2.2. Exhaled breath studies conducted using nose-only tower and flow rate used.

Study Name	Group(G)/ Individual(I)	Sample flow rate (L/min)
Salmonella Mice	G	1.0
Borrelia Balb-C mice 1	I	1.0
Borrelia Balb-C mice 2	I	0.5
Borrelia SCID mice 1	I	1.0
Borrelia SCID mice 2	I	0.5
Endotoxin Mice 1	I	0.5
Endotoxin mice 2	I	0.5
Endotoxin mice 3	I	0.5
Endotoxin mice 4	G	1.0
Endotoxin Rats	I	0.5

Collection of headspace from bio-reactors

For the *Salmonella* feces study, O-ring sealed 550 mL glass bio-reactors were used. A schematic of a bio-reactor is shown in Figure 2.4. For this study, feces samples were mixed with Mili-Q water in a test tube and placed in the bottom of a glass bio-reactor that was sealed. One end of the bio-reactor was then connected via ultra torr connectors and flex tubing to a cylinder of White mountain air and the other end was opened to the room. The air was allowed to flow through the bio-reactor for six minutes at a rate of 1 L/min, after which the flow was stopped and both ends of the bio-reactor were sealed. The bio-reactors were then placed in an incubator set at 37°C.

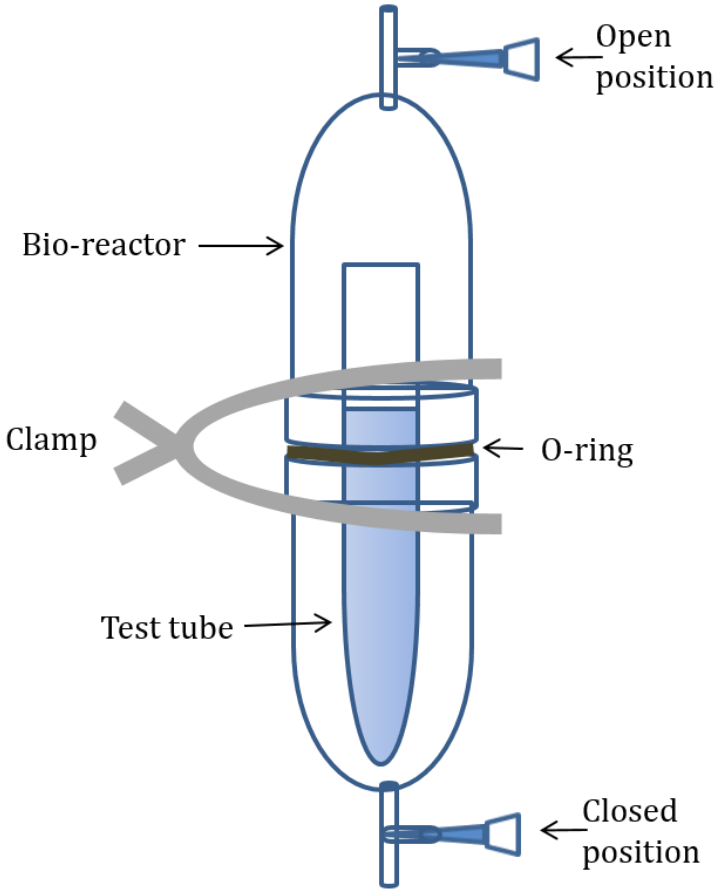

Figure 2.4. Schematic of glass Bio-reactor for feces studies

After incubation the bio-reactors were removed and one end was connected to a manifold containing an evacuated canister connected through a pump. The line through the manifold, between the bio-reactor and the canisters, was sealed to the room and the pump was opened to evacuate this space. Once a vacuum was established the pump was closed and the end of the bio-reactor connected to the line was opened. The sample of headspace was then collected by opening the sample canister. This process was repeated, pumping the line between bio-reactor and canister each time, with a new second canister for each bio-reactor sample. Figure 2.5 shows the bio-reactor connected to a canister through the manifold. A blank sample was also collected from a bio-reactor containing only a test tube and water.

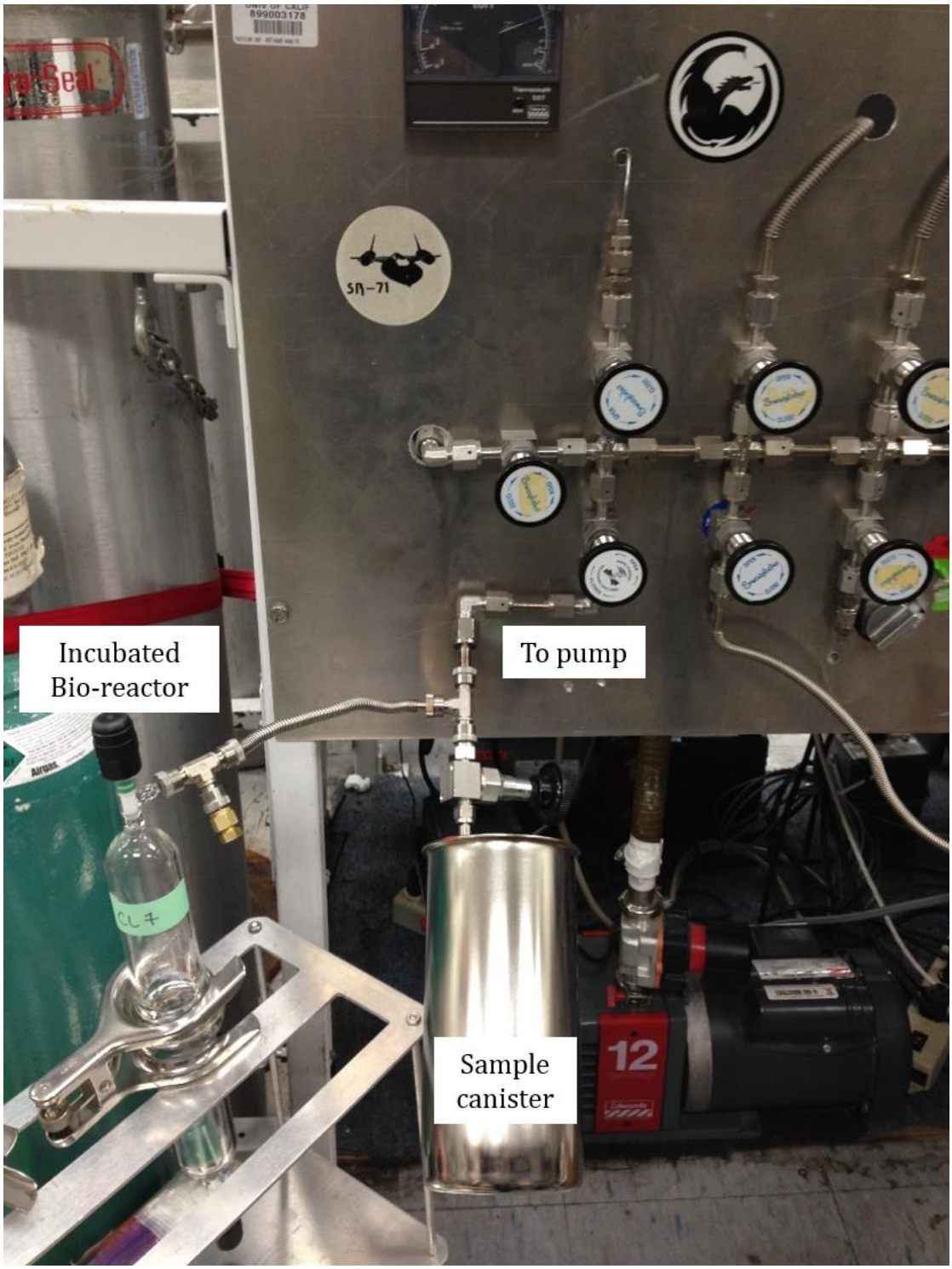

Figure 2.5. Bio-reactor connected to canister through methane manifold for collection of headspace.

Exhaled breath collection from glass bulbs

In addition to the breath collection tower, the Rowland Blake lab has designed two 10 L glass bulbs for the purpose of breath collection from small animals. These glass bulbs, pictured in Figure 2.6, have inlet and outlet stems that were connected to a canister and the White Mountain air cylinder, via stainless steel ultra torr connectors and flex tubing. The animals were then placed inside the bulbs through a large opening that was sealed with an O-ring fitted threaded Teflon ® lid. The bulb was flushed with the White Mountain air for 30 minutes after which the cylinder was closed and the bulb exhaust sealed for an additional 30 minutes, during which the exhaled rat breath mixed with the air from the cylinder. The air from the bulb was then collected by opening the connected evacuated canister before the rat was removed from the cylinder. Blank samples were also collected using the same procedure with empty glass bulbs connected. These samples would differ from the breath samples in that they would also include gases coming from other areas of the rats including bodily fluids and the biota present on the rats.

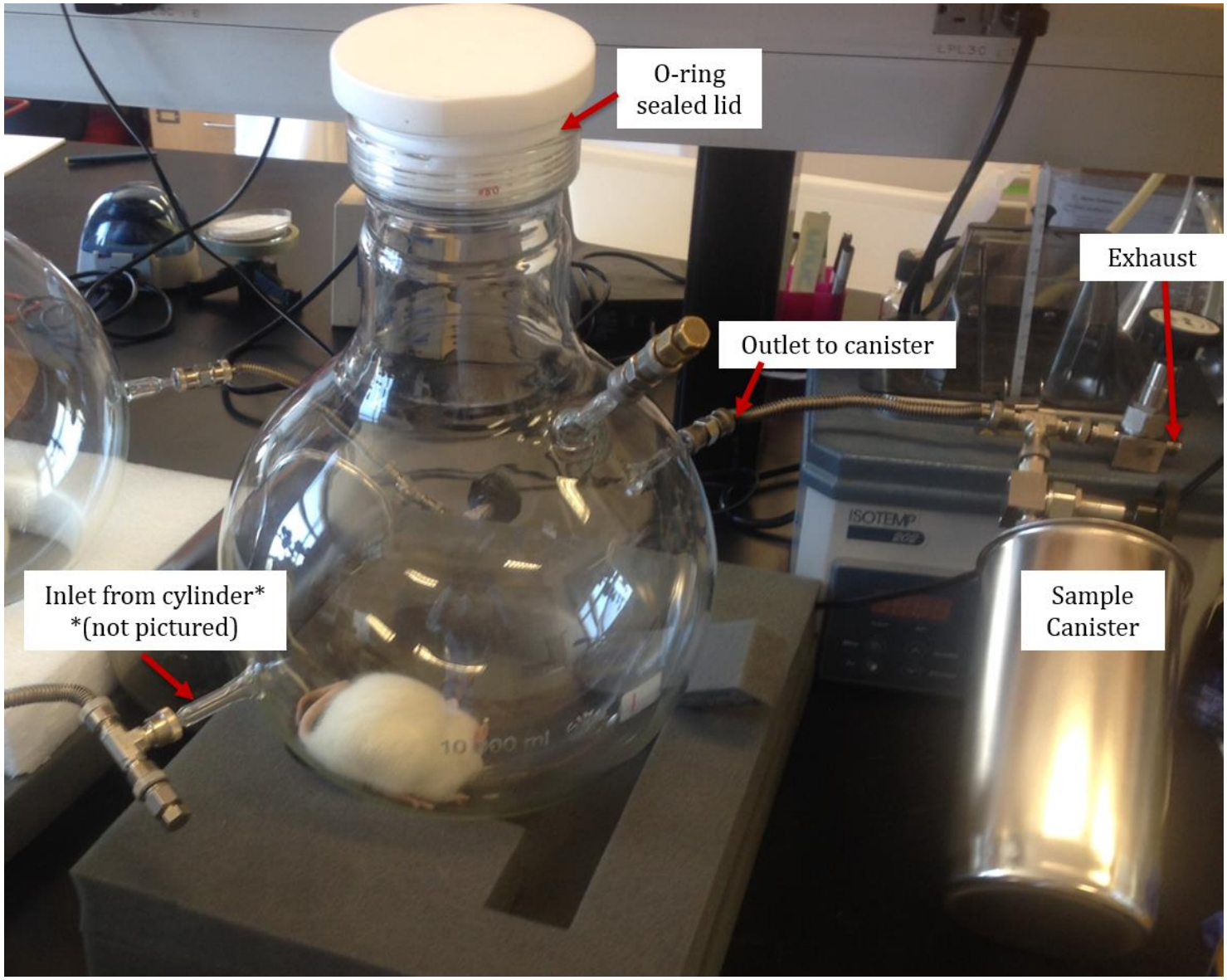

Figure 2.6. Glass bulb breath sampling set up for collection from individual rats.

Sample analysis: CH_4 system

Due to the small volume of the headspace samples, only VOC analysis was performed and therefore the following only applies to breath samples. Methane measurements were made for the breath samples using a Hewlett Packard (HP) model 5890 GC connected to a flame ionization detector (FID). The sample was loaded into a 5 cm^3 injection loop that had been evacuated to at least 10^{-2} torr. Once 400 torr had been loaded, the sample was injected into a 0.9 m 1/8" O.D. packed 80/100 mesh Spherocarb molecular sieve column [3]. The carrier gas used was nitrogen and the detector and oven temperatures were held constant during sampling at 250°C and 85°C, respectively. The FID output was received by a Spectra-Physics® 4400 integrator which gave peak area, retention time, and peak height. The methane peak eluted at 1.1 minute with an accuracy of 1% and precision of 2 ppbv [4, 5].

In addition to the sample canisters, a whole air standard was run prior to each set of eight canisters. The absolute concentration of methane contained in the whole air standard was 1.771 ppm. This amount, along with the averages of the resulting peak areas, was used to calculate a response factor (RF) according to equation 2.1 below.

$$Rf = \frac{Average\ STD\ areas}{Absolute\ STD\ concentraion} \qquad \textbf{(2.1)}$$

This response factor was used to determine the concentration of methane in the samples by dividing the peak area for each sample by the RF calculated from the standards. This gave mixing ratios in ppmv. An example of a chromatogram produced by the methane system is pictured in Figure 2.7.

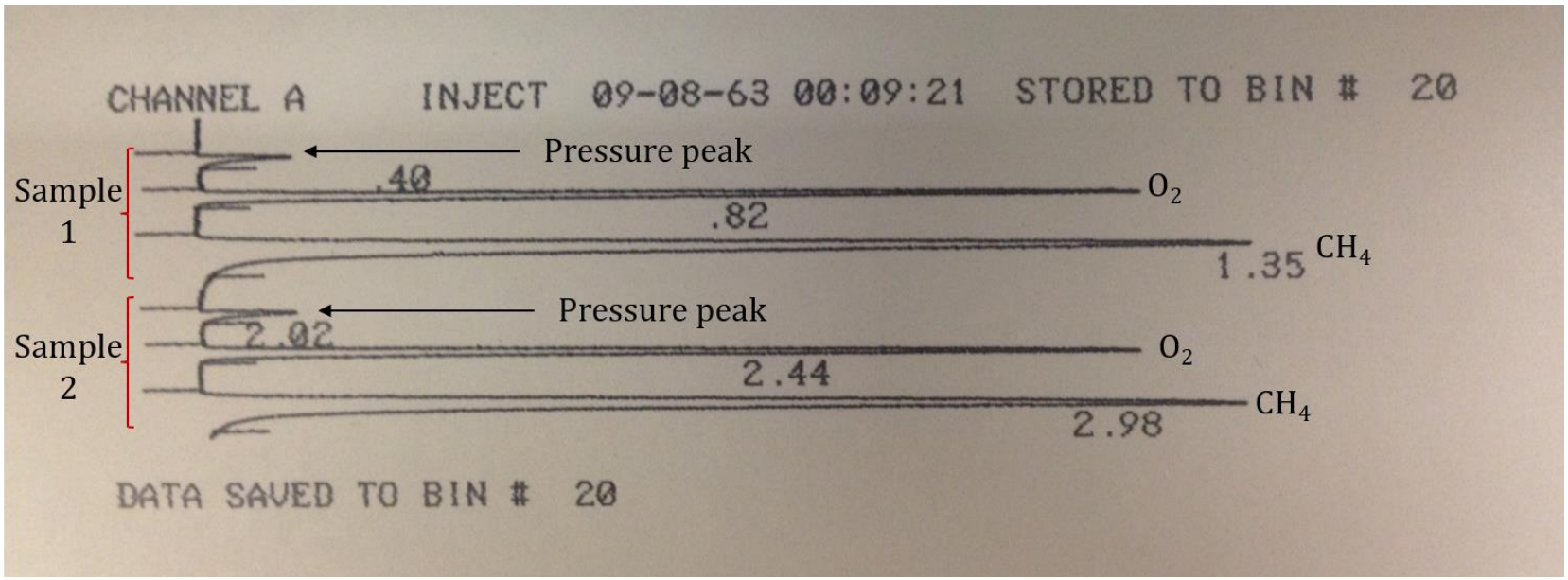

Figure 2.7. Example of chromatogram for methane system showing two sample injections.

As seen in the previous Figure, each sample injection results in three peaks, a pressure peak at around 0.40 min, an oxygen peak at around 0.80 min and finally the methane peak at around 1.35 min. Using the standard in a similar fashion to methane, the relative amount of oxygen in the breath samples was also determined.

CO/CO_2 system

As with the methane system, there was not enough volume to analyze CO or CO_2 in the headspace samples. The first phase of analysis for the collected breath samples was performed using the tandem CO and CO_2 gas chromatography system shown in Figure 2.8. This system consists of a single manifold connecting two gas chromatography oven/detector pairs. Using the manifold, a volume of 10 mL was injected, through a 2.3 cm^3 1/8" stainless steel loop, into each system using helium as a carrier gas in both cases. For CO_2, an 80/100 mesh Carbosphere packed column (1/8" O.D., Alltech) measuring 1.8 meters was connected to a thermal conductivity detector and housed in an oven (HP model 5890) that was increased at 70°C/min from 150°C to 220°C 2.5 min after injection, before returning to the starting temperature for the next sample. The detector was set at 230°C and the retention time was 1.5 min with an accuracy and precision of 5% and 5 ppmv [4, 5]. In order to determine the concentrations, a working standard (STD) was run at the beginning of each set of 8 canisters. The CO_2 concentration in this standard was 364 ppmv which was used to find a response factor (Rf), as discussed for the methane analysis. Similarly, the concentrations of CO_2 in the sample canisters were then calculated by dividing the determined areas by the resulting response factor to give a mixing ratio in ppmv.

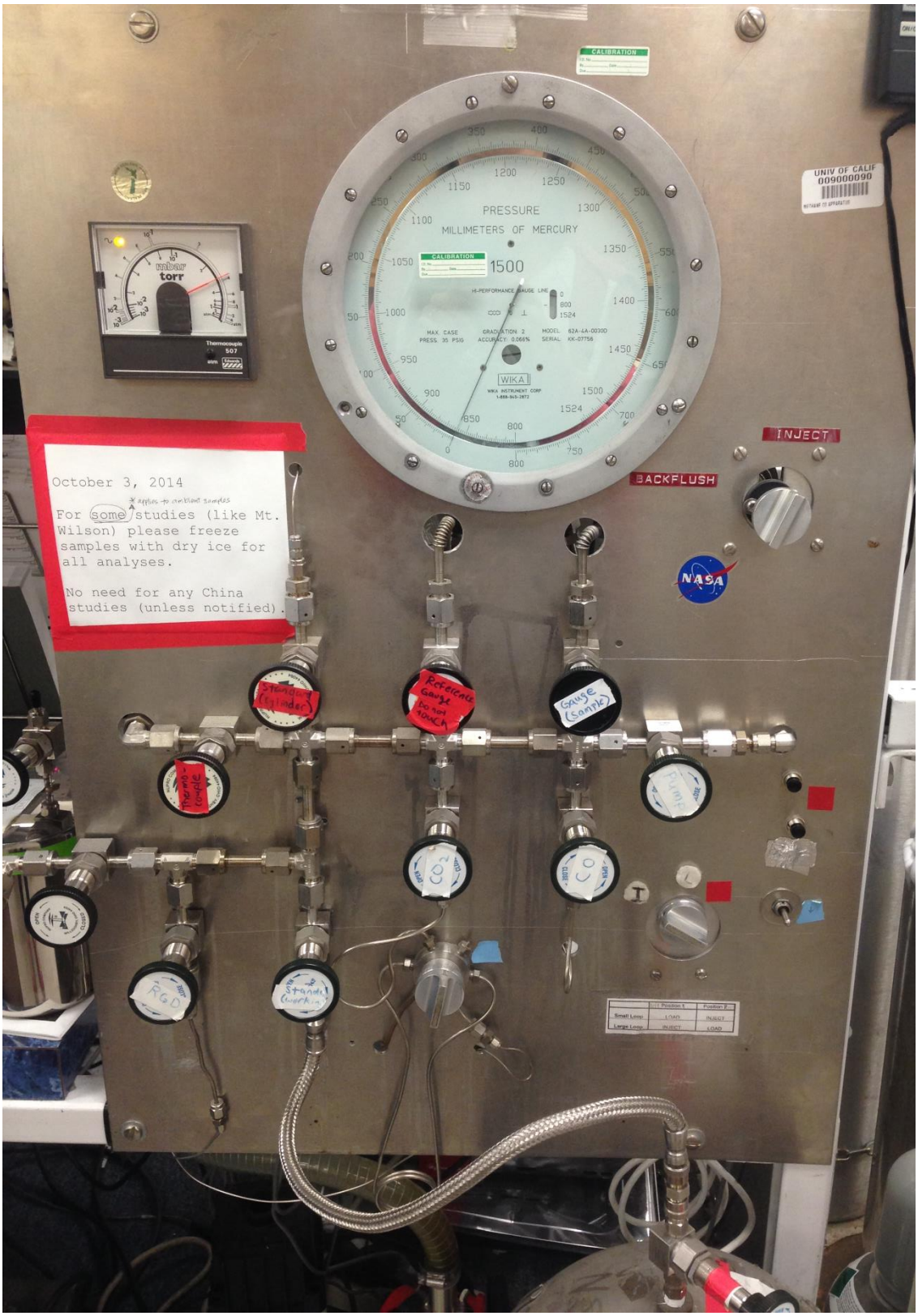

Figure 2.8. Picture of CO/CO_2 manifold.

The CO gas chromatograph (HP 5890) housed a 1/8" O.D. molecular sieve 5A column measuring 3 m which was connected to a flame ionization detector (FID). Prior to introduction to the detector, the CO was converted to CH_4 using H_2 and a nickel catalyst consisting of 2% nickel coating on Chromosorb G (kept at around 370°C), according to equation 2.2 below.

$$\mathbf{3\ H_2 + CO \rightarrow CH_4 + H_2O} \qquad \mathbf{(2.2)}$$

The first 3.5 minutes of the column outflow was vented to the laboratory to prevent oxygen from inactivating the catalyst via oxidation. Next the effluent was diverted to the catalyst using a four way switching valve where it was converted to CH_4, and subsequently detected as CH_4 by the FID, which was set at 250°C. The initial and final temperature for the oven housing the column was 60°C with a 70°C/min increase to 110°C beginning at two minutes and ending at five minutes. The retention time for CO was 5.3 min and accuracy and precision were 5% and 4 ppbv [6, 7]. The CO colujmn was back-flushed with helium for two minutes between samples. The working standard, mentioned in the previous section, also contained 197 ppbv of CO and was used, as in CO_2, to determine sample concentrations via a calculated response factor. A schematic of the CO/CO_2 system summarizing the components is shown in Figure 2.9.

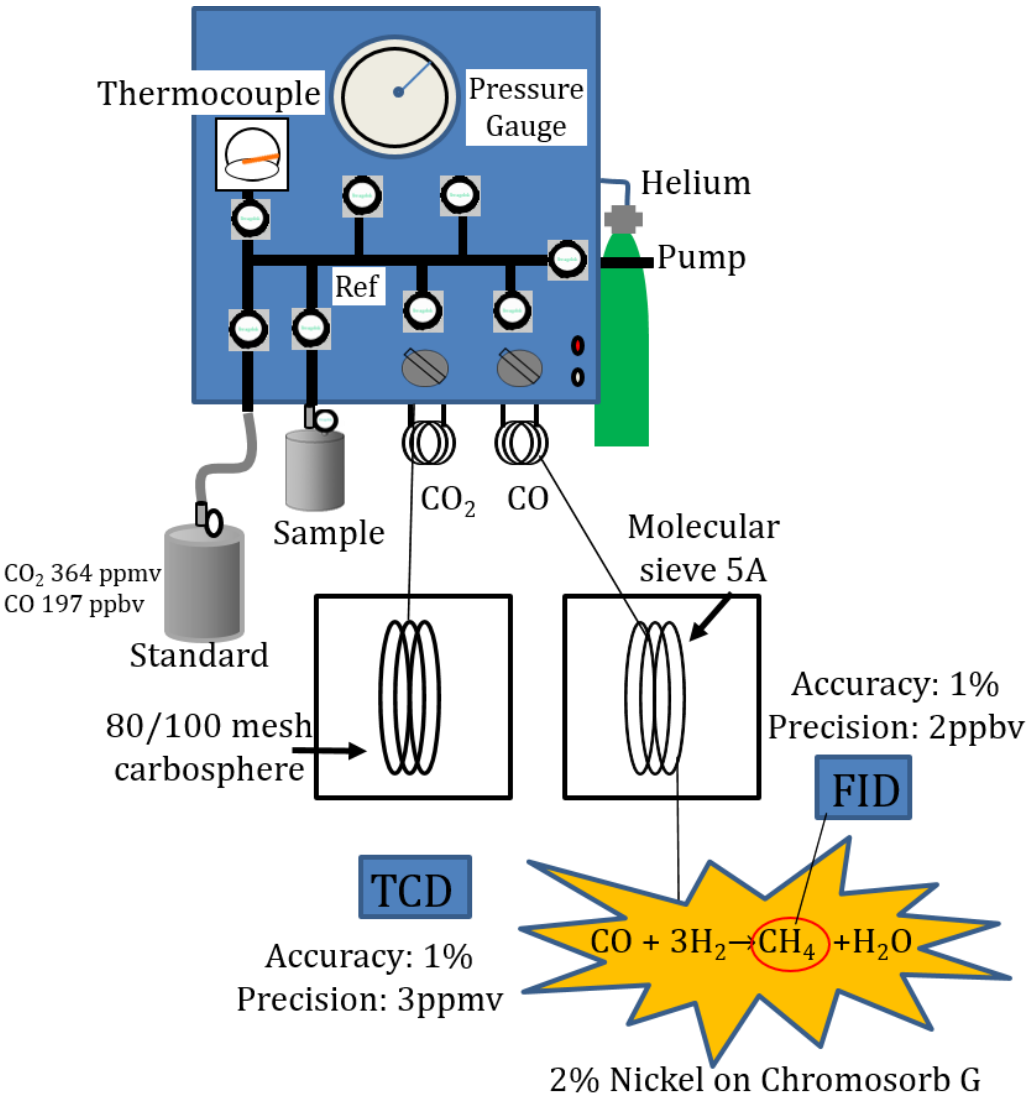

Figure 2.9. Schematic of CO/CO_2 tandem manifold system.

Non-methane hydrocarbon (NMHC) analysis

One major problem with gas chromatography is incomplete separation of gases leading to co-eluting peaks. The multi-column detector system used in the Rowland Blake laboratory solves this issue by employing the use of redundant peaks on multiple columns and detectors. By measuring the same peak on more than one column, the presence of co-eluting peaks can be better identified. This process increases the number of gases that are able to be detected. The sensitivity of the separation is increased by pre-concentration of

the samples before analysis. This step involves cryogenically cooling the sample in a stainless steel loop filled with 3 mm glass beads, to allow lighter gases in the bulk air such as oxygen, nitrogen, and argon, to be pumped away before the sample is re-volatilized and sent to the GCs. A picture of the manifold for this system is shown in Figure 2.10.

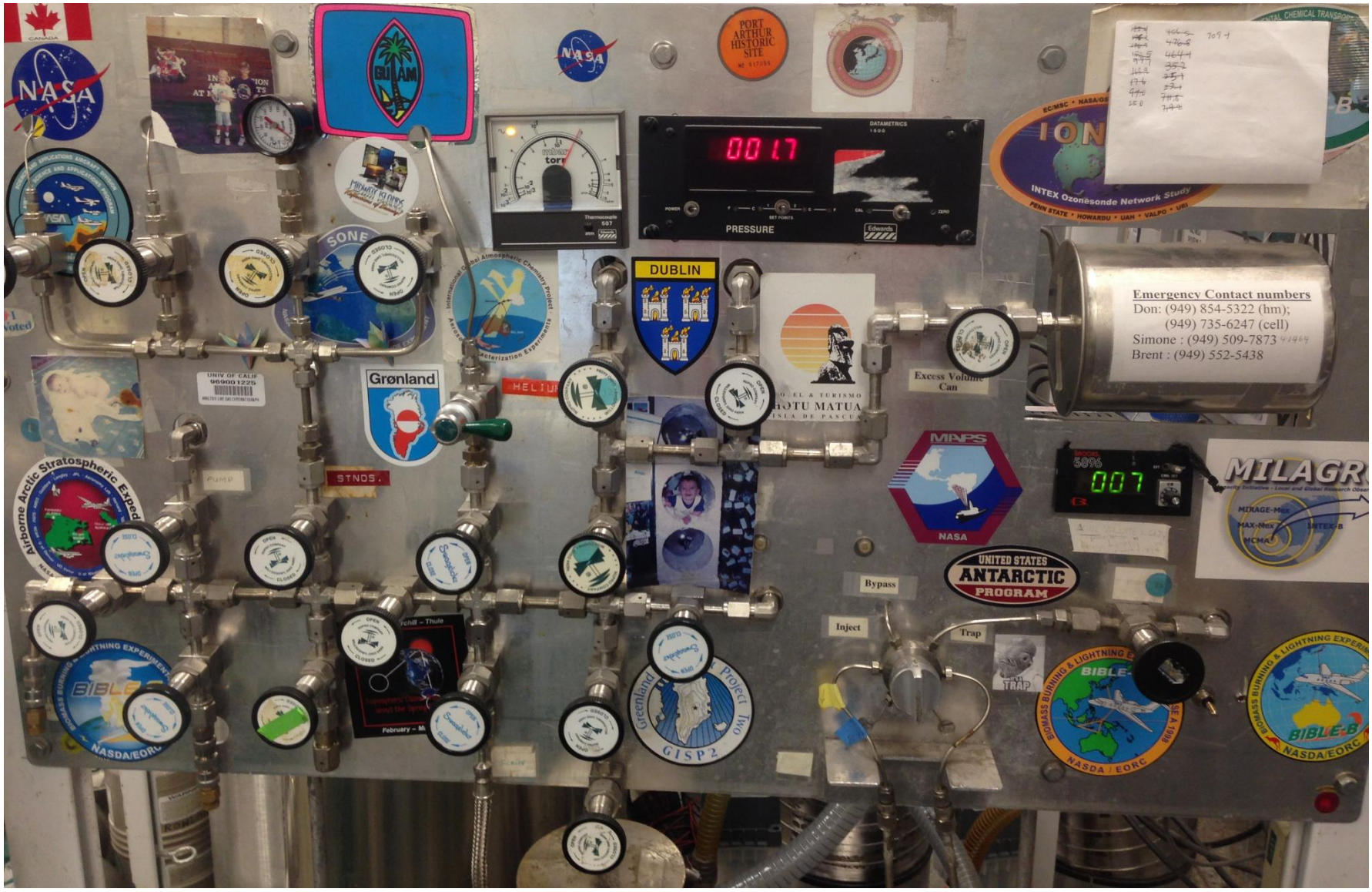

Figure 2.10. Picture of manifold for NMHC analytical system.

For analysis, the samples were connected individually to the NMHC manifold shown in Figure 2.10. As mentioned earlier the first step of analysis for the NMHC system involved cryogenic pre-concentration of the sample in a stainless steel loop by flowing 1.1 L of sample through the trap at a constant flow of 500 mL/min. After this, the sample was isolated in the trap and hot water was placed on the trap to re-volatilize the sample. This was followed by injection of the sample into five separate columns via a six-way splitting valve through a fused silica steel line. The data from each of the five detectors was collected on two computers.

The five separate columns were contained in pairs in a combination of three ovens (Hewlitt Packard 6890 series GC system) each with an independent temperature ramp program. Additionally, the ovens were cryogenically cooled prior to each injection to enhance separation. For identification, the three ovens were given the names GC #2, GC #3 and GCMS. Table 2.3 gives a summary of the oven parameters.

Table 2.3. Summary of 3 GC oven parameters.

	GC #2	**GC #3**	**GCMS**
Carrier Gas	He	He	He
Initial Temp. (°C)	-60	-20	-60
Hold Time (min)	1.5	1.5	1.5
Rate (°C/min)	10	30	15
Final Temp. (°C)	0	60	110
Hold Time (min)	0	0	0
Rate (°C/min)	17	14	29
Final Temp. (°C)	145	220	220
Hold Time (min)	0	3.8	0.95
Rate (°C/min)	65	-	-
Final Temp. (°C)	200	-	-
Total Run Time (min)	17.57	17.96	17.57

The oven for GC #2 contained a single DB-1 column (60 m, 0.320 mm I. D., 1.00 μm film thickness) connected to a flame ionization detector (FID). The DB-1 column is composed of 100% dimethylpolysiloxane and was made by J & W Scientific (Folsom, CA). This column is non-polar, low bleed and has a high temperature limit. The main purpose of this column detector pair was the separation and quantification of C_3-C_{10} hydrocarbons, however several oxygenates were also detected.

The oven for GC #3 housed a pair of columns, the first being a Restek 1701 column (60 m, 0.250 mm I. D., 0.5 μm film thickness) composed of 14% cyanopropylphenyl/86% dimethyl polysiloxane, connected to an electron capture detector (ECD). This combination was used to quantify halocarbons and alkyl nitrates. The second column in GC #3 consisted of an alumina PLOT (30 m, 0.530 mm I. D.), or porous layer open tubular, attached in series to two meters of a DB-1 column (0.320 mm I. D., 1.00 μm film thickness) which was connected to an FID. This combination was used for C_2-C_6 hydrocarbons.

The final oven, GCMS, contained a pair of columns, the first a DB-5 (30 m, 0.250 mm I. D., 1.00 μm film thickness) composed of 5%-Phenyl-methylpolysiloxane attached to a Restek 1701 connected to an ECD. Similar to the column in GC #3, this combination was used to quantify halocarbons and alkyl nitrates, as well as sulfur compounds. The final column-detector combination was a DB-5MS (60 m, 0.250 mm I. D., 1.00 μm film thickness) column connected to a quadrupole mass selective detector (MSD) which was capable of separation and detection of a wide variety of gases including hydrocarbons, halocarbons, alkyl nitrates and sulfur compounds. A schematic of the NMHC analytical system with column detector combinations is shown in Figure 2.11.

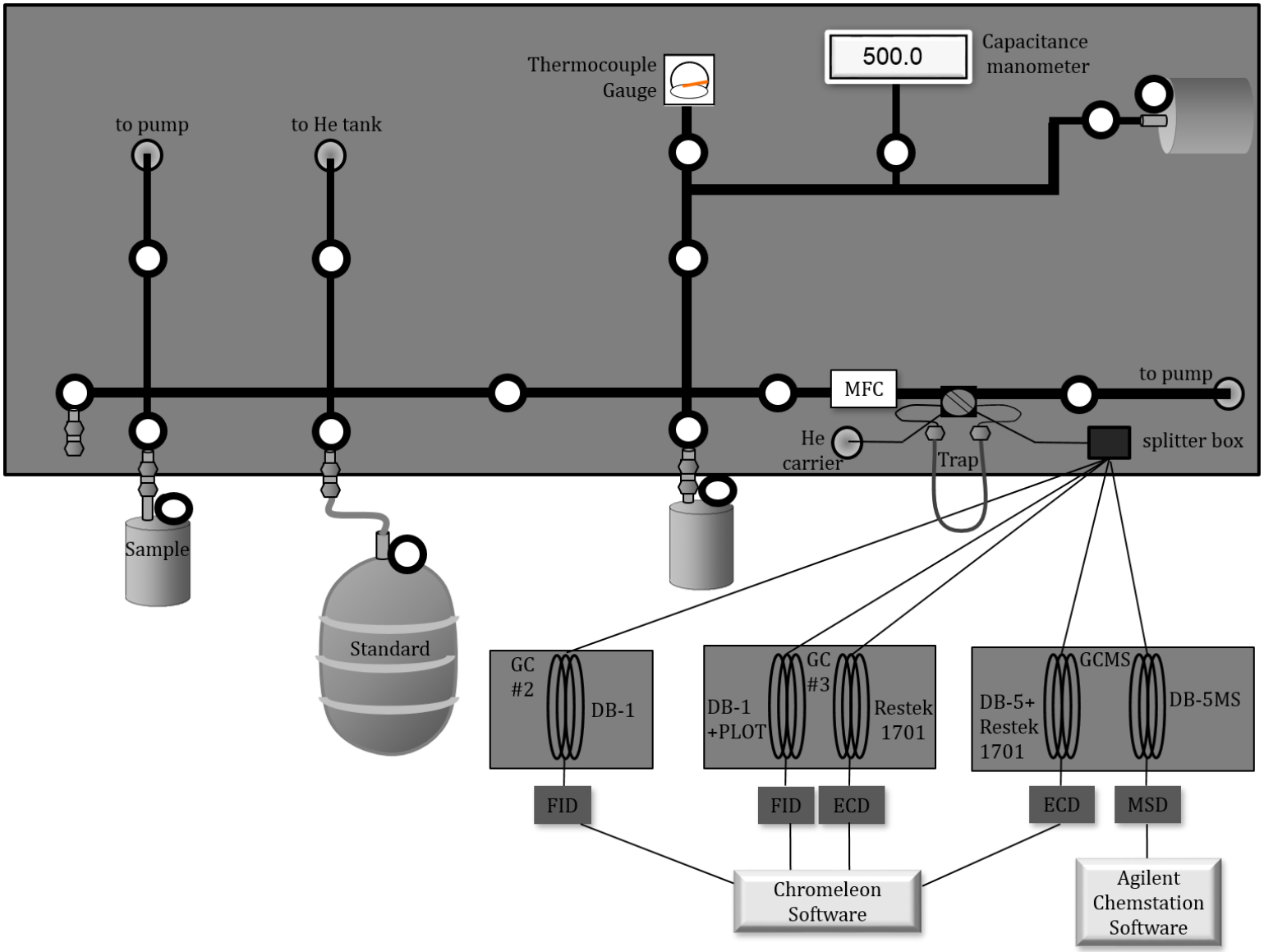

Figure 2.11. Schematic of NMHC analytical system in Rowland Blake laboratory.

Data output from the detectors was recorded for all FIDs and ECDs by a PC using Chromeleon software (Dionex (Thermo Fisher)). The MSD output was recorded by a second PC using the Chemstation software from Agilent. Chromatograms were generated for each column and detector combination by these programs. Figures 2.12.a-d show typical chromatograms for a blank (White Mountain air) and for a rat breath sample for the ECD and FID detectors, respectively.

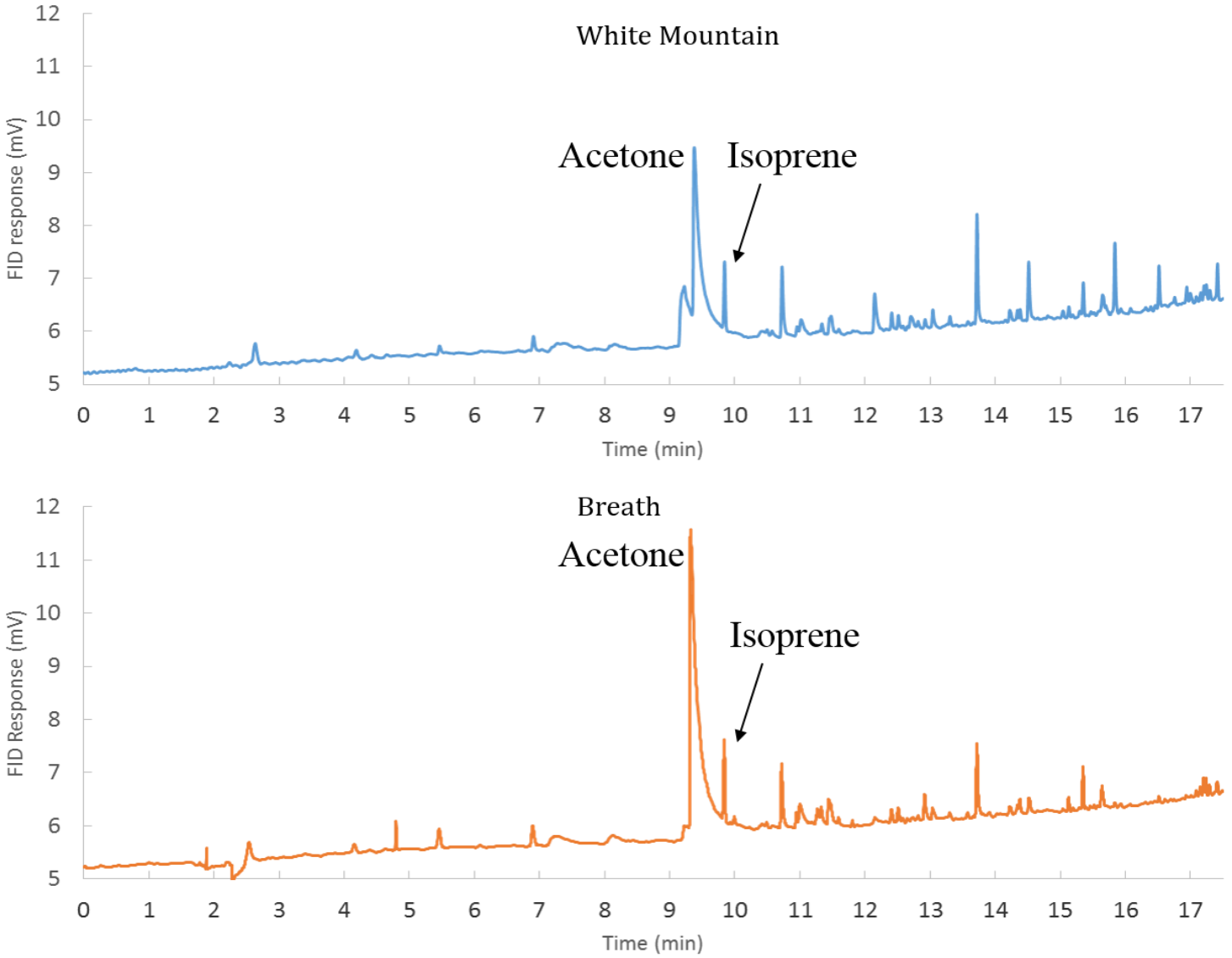

Figure 2.12. a. FID (GC #2) chromatogram for a sample of White Mountain air in breath sample tower (top/blue) and for a breath sample (bottom/orange).

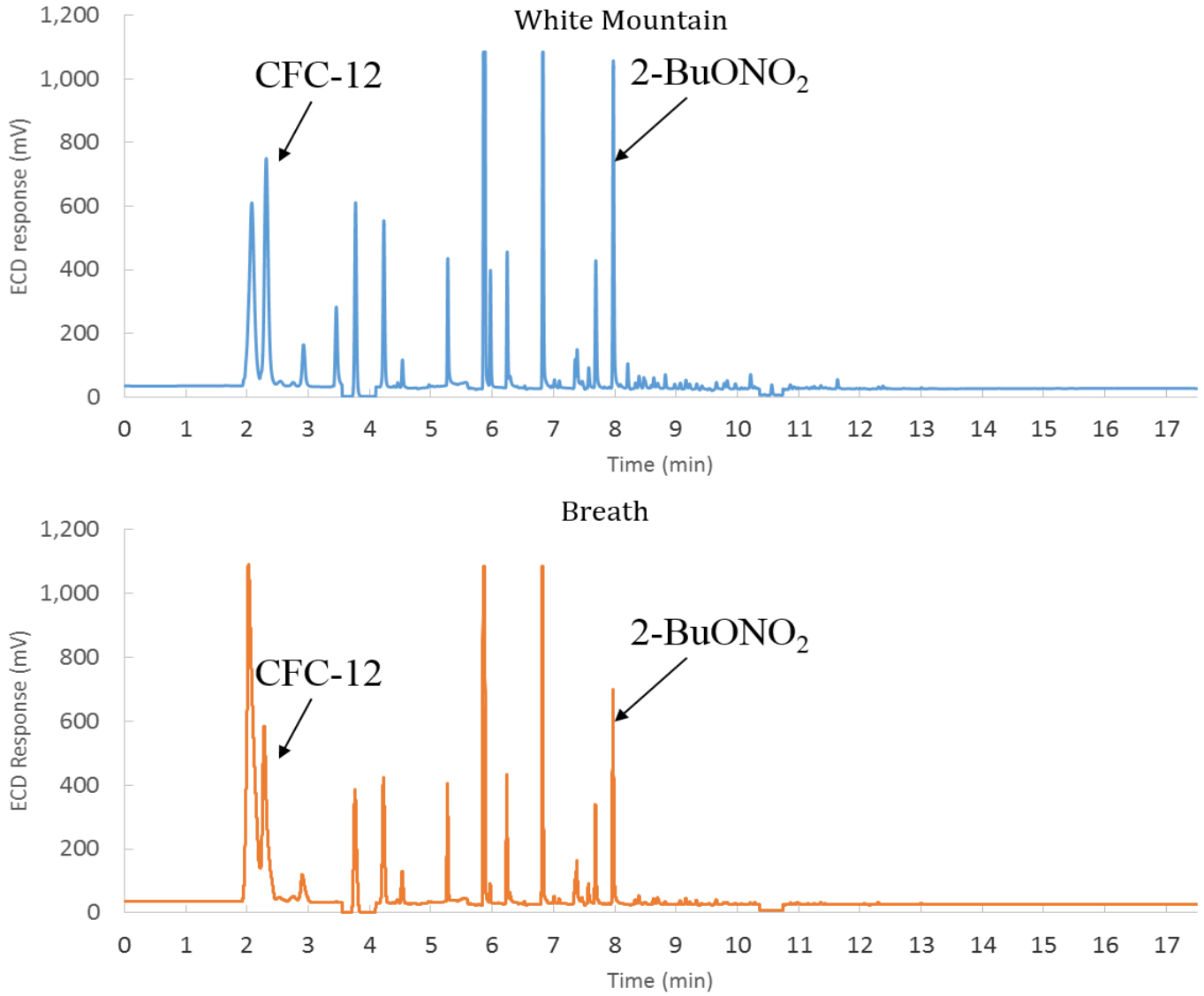

Figure 2.12. b. ECD (GC #3) chromatogram for White Mountain air in breath sample tower (top/blue) and for a breath sample (bottom/orange).

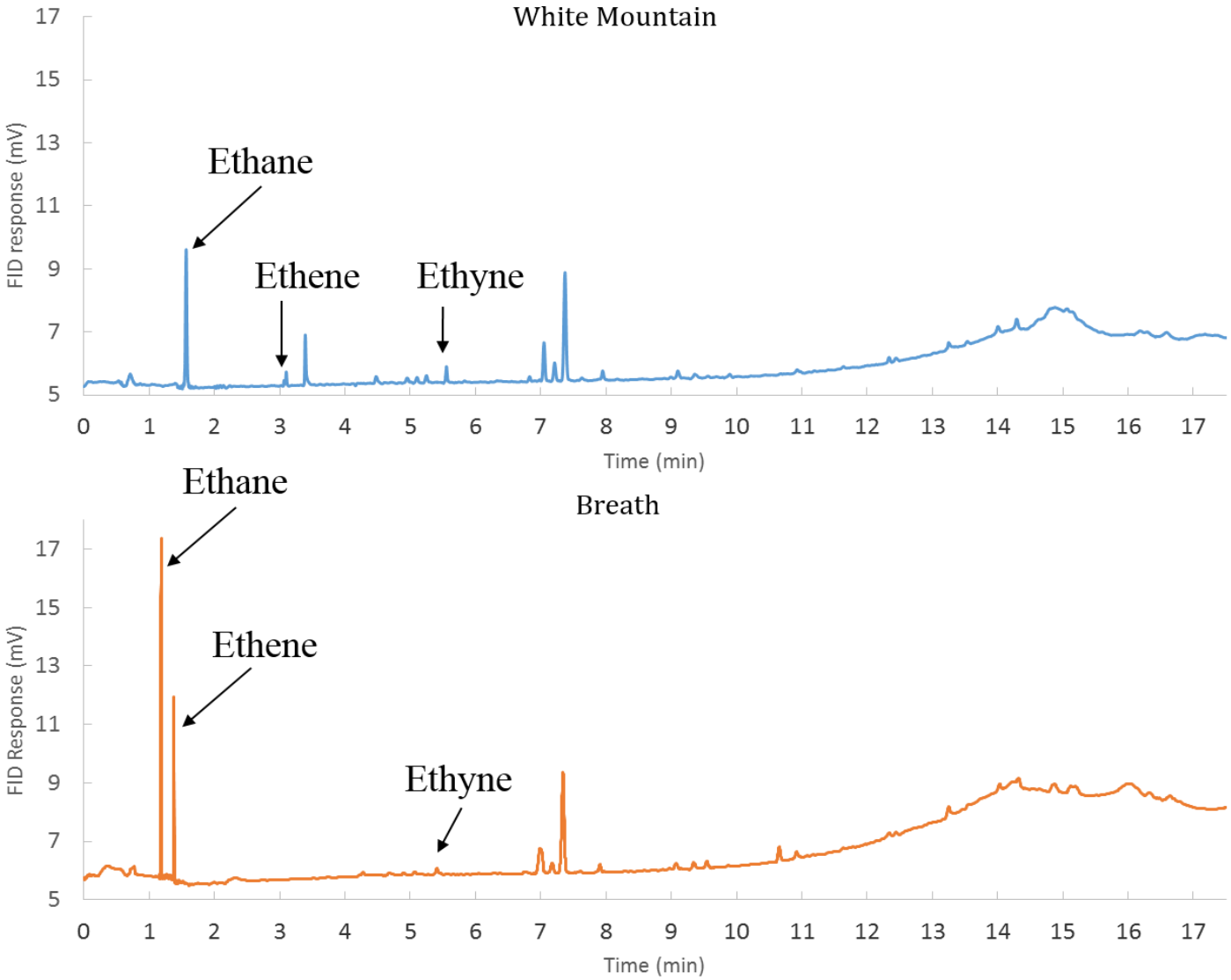

Figure 2.12. c. FID (GC #3) chromatogram for a sample of White Mountain air in breath sample tower (top/blue) and for a breath sample (bottom/orange).

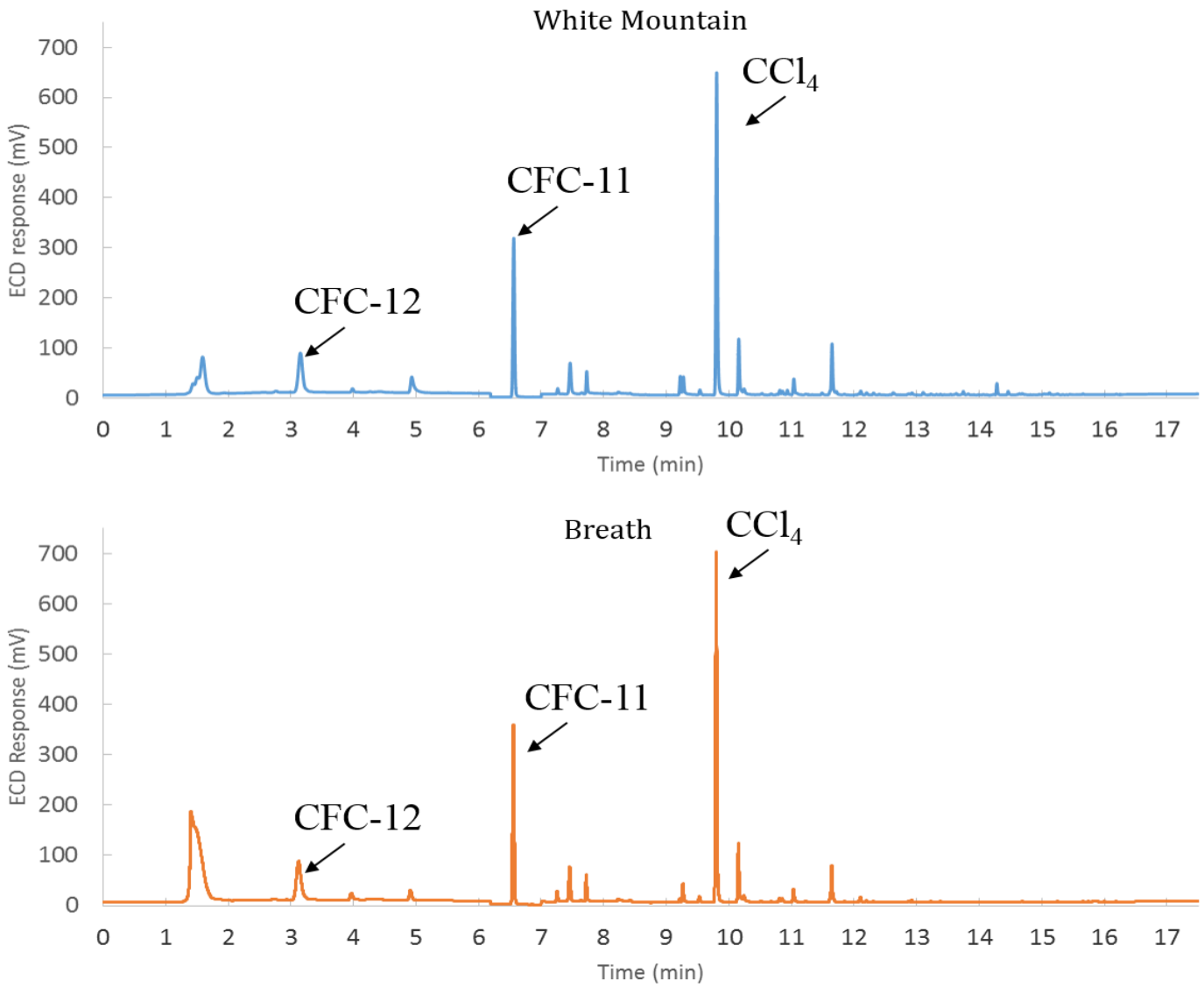

Figure 2.12. d. ECD (GCMS) chromatogram for a sample of white mountain air in breath sample tower (top/blue) and for a breath sample (bottom/orange).

Each peak on the chromatograms corresponds to a specific gas compound. The area under each peak was determined for each sample as well as for whole air standards that were run at the beginning of each set of eight canisters as in the CO/CO_2 and CH_4 analyses. Response factors were calculated using the average areas of the standard divided by the absolute amount contained in the standard for each respective gas measured. The mixing ratios of these gases were then determined by division of the sample area by the calculated response factors.

Collection of White Mountain air

Air was collected in evacuated cylinders from the University of California White Mountain research station which is situated in the Sierra Nevada Mountains at approximately 3,100 meters elevation. In order to fill the cylinders, two dual bellows pumps were connected to inlet lines that were suspended at approximately ten meters above ground. The inlet lines were each split between the two bellows and the outlets were connected to a manifold built for the Rowland Blake laboratory. This manifold is able to connect to two cylinders at a time, with each individually connected to two pump outlets. The cylinders were placed into a foam insert filled with liquid nitrogen before being connected to the pump via the manifold. A specially designed "collar" was placed around the neck of the cylinder and filled with hot water, which was drained and replaced as needed, to prevent the valve from freezing shut while filling. Cylinders were filled individually with one cylinder being filled while the second cylinder was cooling. The collection set-up is show in Figures 2.13a and b.

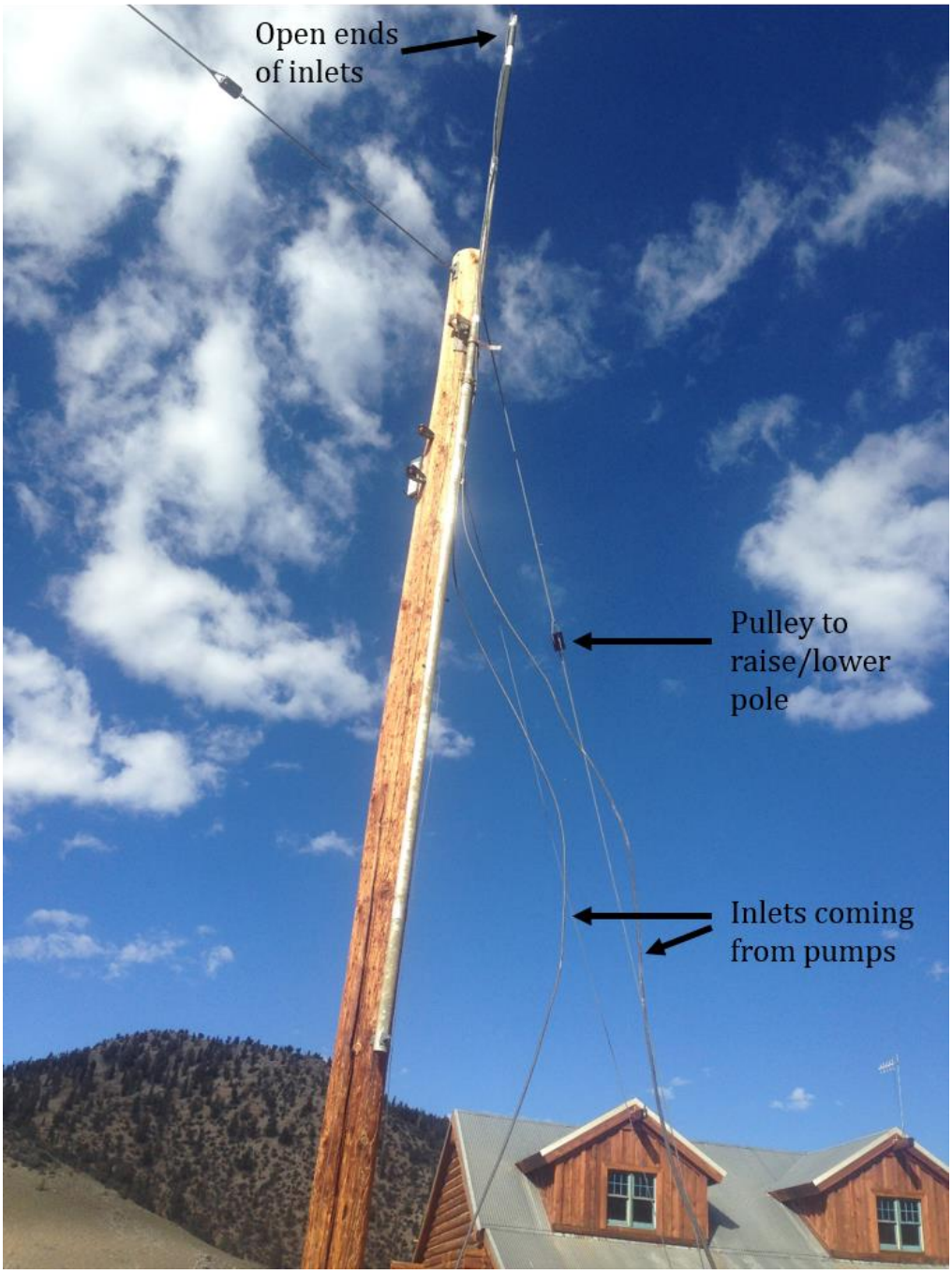

Figure 2.13. a. Picture of suspended pump inlet for cylinder filling.

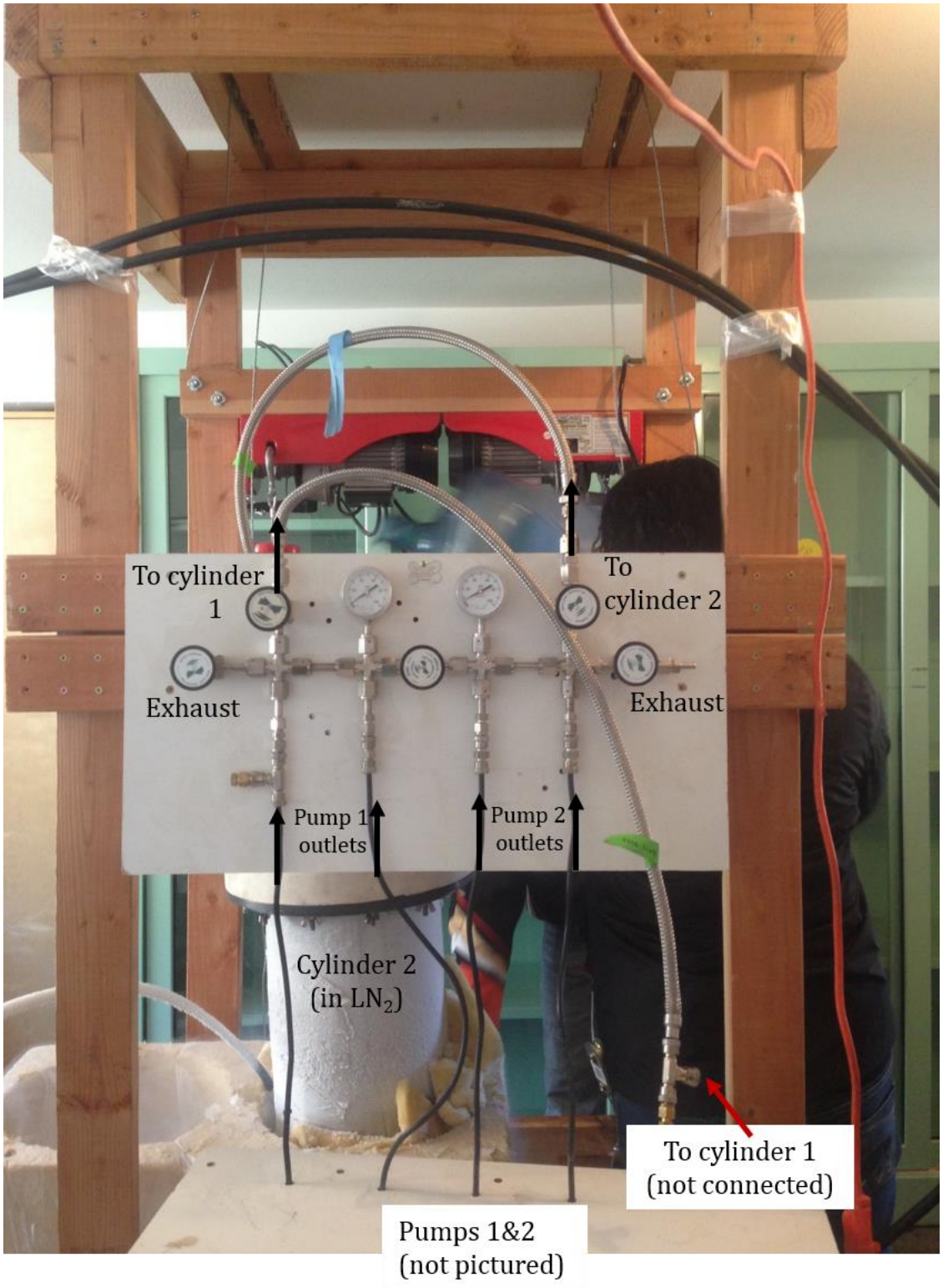

Figure 2.13. b. Picture of manifold with pump and cylinder connected for filling.

The cylinders remained connected to the pump, in the foam insert with liquid nitrogen for approximately four hours. After this time the cylinders were allowed to come to room temperature at which point the pressure was read and recorded. Lastly, the cylinders were inverted and the water inside was blown out by repeatedly cracking the valve in quick bursts until only dry air was released. The pressure was recorded again upon returning to UC Irvine and was around 2000 pounds per square inch (psi) for each cylinder.

Preparation of Standards

The air from the White Mountain cylinders was used for this project in two ways. The first was in pure form during the sampling steps, and the second was during the analysis as the whole air standards. The whole air standards were prepared from White Mountain air according to methods developed by former lab members [8]. Several working standards are used in the Rowland-Blake laboratory including, one for CO/CO_2 analysis, one for CH_4 analysis, and several variations for the NMHC analysis (depending on the project), however all were prepared in a similar fashion. Namely, 43.6 L standard gas cylinders were doped with known amounts of desired gases, for example by adding a mixture of hydrocarbons each at 1 ppmv in nitrogen, then White Mountain air was transferred to the cylinders to about 2,000 psi. This mixture was then added to 34 L electropolished stainless steel pontoons to about 300 psi and 20 torr of water was added to increase stability. All standards prepared in the lab were calibrated against a standard obtained from the National Institute of Standards and Technology (NIST) prior to use for analysis.

Statistics

For all studies, whether breath (from tower or bulb) or a headspace study, blank samples were collected with each set of collections. In the case of the tower, the blank consisted of the air from the White Mountain cylinder being flowed through an empty chamber (for mouse/rat) connected to the tower. Analysis of this sample gave background concentrations of the air the animal would inhale during sampling as well as represented the portion of the sample that diluted the breath during collection which could contain gases produced by the tower. For the bulb study, the blank consisted of air from the White Mountain cylinder that had been sealed in an empty bulb for the same amount of time as the rat and was collected in the same manner as the breath sample. This would represent the White Mountain air from the cylinder as well as indicate if any gases were produced by the bulb over time. The headspace blanks were collected from a bio-reactor that contained a test tube with the same type and amount of water as the feces samples had been mixed with. This blank gave an indication of any gases that could have come from the bio-reactor, test tube, or water during incubation.

In order to account for the gases present in the background air in the breath (or headspace) samples, the sample concentrations were corrected by taking the difference between the measured concentration and the blank concentration for each gas in each respective sample. These corrected samples will be referred to as corrected "VOC" concentration or simply as corr ["VOC"]. An example is shown in equation 2.3.

$$\textbf{Corr } CO_2\% = CO_2\%_{\textbf{sample}} - CO_2\%_{\textbf{blank}} \qquad \textbf{(2.3)}$$

Additionally, initial studies indicated that dilution of the breath samples collected from the tower was enhanced in certain samples. The enhancement of the dilution was

predominantly seen in sick animal samples, likely due to slower shallower breathing by the animal. This slower and shallower breathing by the animal would lead to less sample volume having been exchanged with the lungs and therefore a smaller percentage of breath would be represented by the sample. An example of this is shown in Table 2.4 which uses average breathing rates of healthy and infected mice along with average lung volume to determine the differences in the amount of breath that could be collected [9].

Table 2.4. Example of differences in amount of breath sample collected from healthy vs. infected mice [9].

	Uninfected	**Infected**
Breathing rate (bpm)	350	200
Volume/breath (mL)	0.14	0.070
Breath vol./time (L/min)	0.049	0.014
Breath vol. per sample* (L)	0.20	0.056
% Breath per 2L sample	10	2.8

*assuming 4 minute sample time

To account for the fact that the "amount" of breath collected would vary for collected breath samples, CO_2 a gas well-known to be present in breath and which is present in concentrations much higher than in the blank air, was used. The amount of CO_2 determined for each sample was taken to represent the amount of breath in the sample and used by division of the measured gas concentration by the $\%CO_2$. This normalized each of the collected samples to improve the statistical analysis. To test the null hypothesis that there was no significant difference between the means of samples of infected and uninfected mice (or rats), the probability values (p-values) were calculated from the Student's t-test [10, 11]. For the t-test, an F-test was first used to determine if variance was equal or unequal, the results of which would indicate the t-test used. The alpha value was 5%.

References

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Chapter 3: *Salmonella Enterica*

Introduction

Salmonella enterica is a Gram-negative bacterium that causes an infection termed salmonellosis [1]. This infection is commonly caused by contaminated food but can also be spread by poor hygiene, contaminated water, and contact with certain animals and reptiles [2]. While the infection usually progresses without the need for medical intervention in healthy adults, in certain persons including the elderly and immunocompromised individuals the infection can require hospitalization and even be fatal. Between 1998 and 2008 the CDC reported 1491 outbreaks of salmonellosis [3]. In the United States alone, there are roughly 400 deaths per year and as many as 1.2 million reported infections attributed to salmonellosis [4]. The economic impact of these cases can be attributed to a loss of more than $4.4 billion per year [5]. Two sub-species of *Salmonella enterica* have been noted as the first and second most common causes of *Salmonella* outbreak, together accounting for over 60% of all *Salmonella* infections worldwide [6]. For these reasons, a tool for the rapid detection and monitoring of *Salmonella enterica* infection would be a great asset to the global community.

Methods: Feces headspace

For this study, nine samples of feces collected from two types of mice were obtained from Dr. Raffatellu's laboratory at UCI. Of these samples, three were from wild-type uninfected mice, three were from wild-type mice infected with *Salmonella enterica*, and three were from genetically modified knock-out mice infected with *Salmonella enterica.* Each sample was weighed, placed in a labeled glass test tube and 10 mL of nano-pure water was added. The samples were stirred to dissolve then placed individually into glass bio-

reactors. The bio-reactors were flushed with White Mountain air for six minutes at a rate of 1 L/min after which they were sealed and placed in an incubator at 37°C. After one hour of incubation, the headspace was collected from each bio-reactor which was then flushed with White Mountain air before being incubated a second time for 24 hours. The headspace was again collected from the bio-reactors after this second incubation period. Blank samples were also prepared and collected using the same procedure with 10 mL of nano-pure water in a test tube with no feces. All samples were analyzed for NMHC's in the Rolwand Blake lab.

Methods: *Salmonella* breath study

In this study, four cages containing three mice each were obtained from Dr. Raffatellu's lab. Over three days, breath samples were collected from the mice grouped by cage using the exhaled breath tower. During this initial period, all mice were uninfected. After sampling on the second day, the mice received an injection of the antibiotic Streptomycin. After the third day of sampling, the mice in three of the four cages were infected with *Salmonella enterica.* After the infection day, samples were collected daily for four consecutive days. For unknown reasons, a mouse in the uninfected cage, designated cage number four, expired on the second day of infected sampling. Prior to sampling on the third day of infected sampling a mouse in one of the infected cages, designated cage number three, also expired. In addition to the samples from the groups of mice, blank samples were collected at the beginning of each sampling day using three empty restraint tubes attached to the tower.

Results: Breath CO, CO_2, CH_4, and O_2

The breath samples were analyzed for concentrations of CO, CO_2, CH_4, and O_2 in the Rowland Blake lab to determine if any differences would be present throughout the course of the infection. The results of these measurements are summarized in Table 3.1.

Table 3.1. Concentrations of CH_4, O_2, CO, and CO_2 in breath of mice.

CAN #	Cage #	Status	Day	CH4 (ppmv)	Oxygen (%)	CO (ppbv)	CO2 (%)
1325	0	BLANK	-3	1.816	20.2	104	0.04
1032	1	UNINFECTED	-3	1.843	19.8	120	0.28
504	2	UNINFECTED	-3	1.829	19.8	119	0.27
1410	3	UNINFECTED	-3	1.841	20.1	106	0.15
2420	4	UNINFECTED	-3	1.835	19.9	114	0.23
403	0	BLANK	-2	1.871	20.2	128	0.04
710	1	UNINFECTED	-2	1.848	18.8	138	0.82
1413	2	UNINFECTED	-2	1.827	19.2	117	0.52
921	3	UNINFECTED	-2	1.850	18.9	148	0.72
1201	4	UNINFECTED	-2	1.831	18.9	111	0.67
1525	0	BLANK	-1	1.831	20.3	93	0.04
1225	1	UNINFECTED	-1	1.822	19.0	125	0.65
2423	2	UNINFECTED	-1	1.833	19.0	140	0.71
428	3	UNINFECTED	-1	1.824	19.1	138	0.63
313	4	UNINFECTED	-1	1.801	18.8	132	0.70
232	0	BLANK	1	1.821	20.3	93	0.04
1202	1	INFECTED	1	1.833	19.3	130	0.62
2402	2	INFECTED	1	1.840	19.0	137	0.68
401	3	INFECTED	1	1.825	19.5	143	0.52
1420	4	UNINFECTED	1	1.834	19.2	134	0.61
1917	0	BLANK	2	1.837	20.4	97	0.04
306	1	INFECTED	2	1.842	19.4	116	0.51
2304	2	INFECTED	2	1.838	19.7	124	0.35
220	3	INFECTED	2	1.837	19.3	126	0.60
1898	4	UNINFECTED	2	1.817	19.5	121	0.43*
716	0	BLANK	3	1.828	20.3	96	0.04
825	1	INFECTED	3	1.837	19.0	125	0.66
1027	2	INFECTED	3	1.837	19.1	127	0.64
1827	3	INFECTED	3	1.820	19.8	107	0.29*
1105	4	UNINFECTED	3	1.835	19.3	120	0.62*
1824	0	BLANK	4	1.833	20.2	96	0.04
1708	1	INFECTED	4	1.830	19.5	124	0.40
2401	2	INFECTED	4	1.828	19.3	127	0.47
2532	3	INFECTED	4	1.834	19.7	116	0.21*
1623	4	UNINFECTED	4	1.840	19.0	134	0.69*

*Only two mice per cage for these samples

The flow of White Mountain air was measured prior to the start of sampling each day. On the first day of sampling (Day -3), the flow was set at 1.25 L/min while on subsequent sampling days, the flow was reduced to 1 L/min. The effect of this reduction in flow rate is evidenced by the resulting CO_2 concentrations in the above Table, specifically the overall higher concentrations obtained after the first day of sampling. The resulting lower CO_2 values are shown in red for the first day of sampling, again the result of the increased flow rate of background air leading to higher dilution of the breath samples. Because of this the samples from this day were not included in the following analyses. As mentioned previously, a mouse from each of cages three and four expired prior to the end of the study leaving only two mice per group in those cases. These samples are identified by a star next to the CO_2 values.

The CO_2 concentrations on the last day of sampling (Day 4) were found to be higher in the uninfected cage, which now only contained two mice. This was higher than in any of the infected cage samples, including the two cages with three mice per sample. This would suggest that the infected mice were breathing less, both slower and more shallow, leading to increased dilution of the breath samples for these mice. This difference in respiration can be accounted for by division of the concentration of CO_2 for each sample. Additionally, the samples were further corrected by subtraction of the blank concentration for each gas, prior to division of CO_2. The resulting concentrations were averaged and a t-test was performed to determine if any significant difference was found. The results of these analyses is shown in Tables 3.2 a. and b.

Table 3.2 a. Results of CH_4, O_2, CO, and CO_2 after subtraction of blanks.

CAN #	Cage #	Status	Date	CH_4 (Corr)	Oxygen (Corr)	CO (Corr)	CO_2 (Corr)
710	1	UNINFECTED	-2	-0.024	-1.421	10.0	0.78
1413	2	UNINFECTED	-2	-0.044	-1.023	-10.4	0.48
921	3	UNINFECTED	-2	-0.022	-1.255	20.5	0.68
1201	4	UNINFECTED	-2	-0.041	-1.288	-16.2	0.63
1225	1	UNINFECTED	-1	-0.009	-1.307	32.0	0.61
2423	2	UNINFECTED	-1	0.001	-1.328	47.5	0.67
428	3	UNINFECTED	-1	-0.007	-1.211	45.3	0.59
313	4	UNINFECTED	-1	-0.030	-1.512	39.5	0.66
1202	1	INFECTED	1	0.012	-1.017	37.2	0.59
2402	2	INFECTED	1	0.019	-1.309	44.6	0.65
401	3	INFECTED	1	0.004	-0.883	49.9	0.49
1420	4	UNINFECTED	1	0.013	-1.114	41.0	0.58
306	1	INFECTED	2	0.005	-0.955	18.7	0.47
2304	2	INFECTED	2	0.001	-0.654	27.2	0.32
220	3	INFECTED	2	0.000	-1.096	29.1	0.56
1898	4	UNINFECTED	2	-0.020	-0.878	24.0	0.39
825	1	INFECTED	3	0.009	-1.262	29.4	0.62
1027	2	INFECTED	3	0.009	-1.246	31.2	0.60
1827	3	INFECTED	3	-0.007	-0.526	11.6	0.25
1105	4	UNINFECTED	3	0.007	-1.055	23.9	0.58
1708	1	INFECTED	4	-0.003	-0.634	28.4	0.36
2401	2	INFECTED	4	-0.005	-0.889	31.4	0.44
2532	3	INFECTED	4	0.001	-0.435	20.4	0.18
1623	4	UNINFECTED	4	0.007	-1.177	38.1	0.66

Table 3.2 b. Corrected concentrations of CH_4, O_2, and CO divided by CO_2, averaged for t-test.

Status	Date	CH_4 (Corr)/CO_2	Oxygen (Corr)/CO_2	CO (Corr)/CO_2
UNINFECTED (avg)	-1	-0.0174	-2.12	65.2
UNINFECTED	1	0.0231	-1.93	71.0
UNINFECTED	2	-0.0507	-2.24	61.4
UNINFECTED	3	0.0127	-1.81	41.0
UNINFECTED	4	0.0107	-1.79	58.0
INFECTED (avg)	1	0.0200	-1.86	78.2
INFECTED (avg)	2	0.0045	-2.02	59.3
INFECTED (avg)	3	0.0003	-2.06	48.3
INFECTED (avg)	4	-0.0040	-2.10	89.3
	ttest	**0.28**	**0.40**	**0.19**

As seen in Tables 3.1 and 3.2 a, the blank on Day -2 had an abnormally high concentration of CO and was therefore excluded from the t-test analysis. The results of the t-test however showed no significant difference (p-value > 0.05) between the uninfected and infected samples over the course of the sampling. If however the t-test was performed on all uninfected samples compared to the infected samples on only the last day of sampling, on which day the mice would be presumed to be the sickest, the p-value for the CO (Corr)/CO_2 was 0.02 which would be considered significant. This could mean that if sampling had continued beyond the last day a more significant effect of infection may have been observed. It should be noted however that the t-test is ineffective on small sample sizes, and therefore cannot be reliably used in this way. The small sample size and short sampling period for this study suggests that further experiments should be performed before significant conclusions can be drawn.

Results: NMHC analysis for breath samples.

In addition to the above gases, all collected breath samples were analyzed for NMHC concentration using the Rowland Blake gas chromatography system. The resulting chromatograms were analyzed to produce spreadsheets with the various concentrations determined. The data was analyzed to determine if differences existed between the uninfected and infected samples throughout the study. As mentioned in the section above, days -3 and -2 were found to have significant differences because of sampling error and were excluded from further analysis for this section as well. For this reason, the first day of sampling was considered to be day -1 with infection occurring at the end of this sampling day.

T-tests were performed comparing the data in two ways, as above. First, all infected and uninfected data, starting on day -1, were compared however this showed no significant difference for any gases. Next the uninfected samples from day -1 through day 4 were compared to the infected samples from the last day (day 4) and at this point three possible gases were identified. The data were analyzed both in the raw form and after subtracting the blank values then dividing by the CO_2 concentration (%). The three gases and their corresponding t-test values are shown in Table 3.3.

Table 3.3. P-values for gases identified as significant on the last day of sampling by t-test.

		i-Propanol	Acetone	Unknown
Raw data	**All infected**	0.09	0.09	0.07
Corrected	**All infected**	0.07	0.09	0.18
Raw data	**Last day**	6.6E-04	<E-04	8.6E-03
Corrected	**Last day**	1.7E-04	<E-04	2.4E-02

In order to further examine the differences between the uninfected and infected data, the averages of the infected samples were determined for each of the infection days (1-4). The concentrations of each gas for the uninfected samples were then subtracted from these averages to determine the delta value for each day. This was done for both the raw and corrected data and the results are shown in Tables 3.4. a and b. For the raw data the average blank values for days 1 through 4 are also listed.

Table 3.4. a. Uninfected and average infected concentrations for infection sampling days with calculated delta values for raw data.

Raw data	i-Propanol (ppbv)	Acetone (ppbv)	Unknown gas (Area unit)
Average Blank	0.05	4.8	158
Uninfected day 1	1.84	6.4	136
Uninfected day 2	0.34	8.6	229
Uninfected day 3	0.82	3.4	61
Uninfected day 4	4.37	5.2	141
Infected (avg) day 1	0.41	5.5	163
Infected (avg) day 2	0.99	5.5	196
Infected (avg) day 3	4.10	3.7	274
Infected (avg) day 4	30.44	36.7	712
Delta day 1	-1.43	-0.9	28
Delta day 2	0.66	-3.1	-33
Delta day 3	3.28	0.3	212
Delta day 4	26.08	31.5	571

Table 3.4. b. Uninfected and average infected concentrations for infection sampling days with calculated delta values for corrected data.

Corrected	i-Propanol (ppbv/%)	Acetone (ppbv/%)	Unknown gas (Area unit/%)
Uninfected day 1	3.2	1.4	-91
Uninfected day 2	0.7	13.2	274
Uninfected day 3	1.1	-3.3	-290
Uninfected day 4	6.7	0.4	78
Infected (avg) day 1	0.7	-0.6	-54
Infected (avg) day 2	1.9	5.0	153
Infected (avg) day 3	11.7	-3.0	25
Infected (avg) day 4	100.9	100.3	2933
Delta day 1	-2.5	-2.0	37
Delta day 2	1.1	-8.3	-121
Delta day 3	10.6	0.3	315
Delta day 4	94.2	100.0	2855

In order to visualize the differences between the uninfected and infected samples, graphs were made for the raw and corrected data. The concentrations of the above gases were plotted by day (-1 to 4) and colored by identity. The blue was chosen to represent the blank samples, green for uninfected samples and red for the infected samples. The resulting graphs are shown in Figures 3.1. a, b, and c.

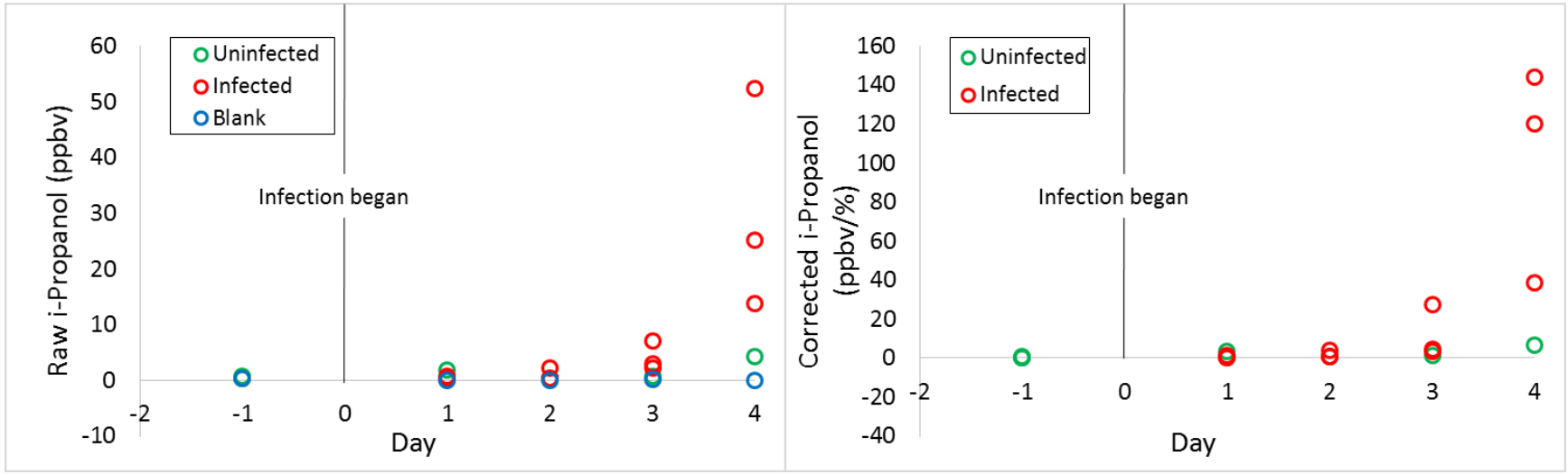

Figure 3.1. a. Graphs of raw and corrected data for i-propanol.

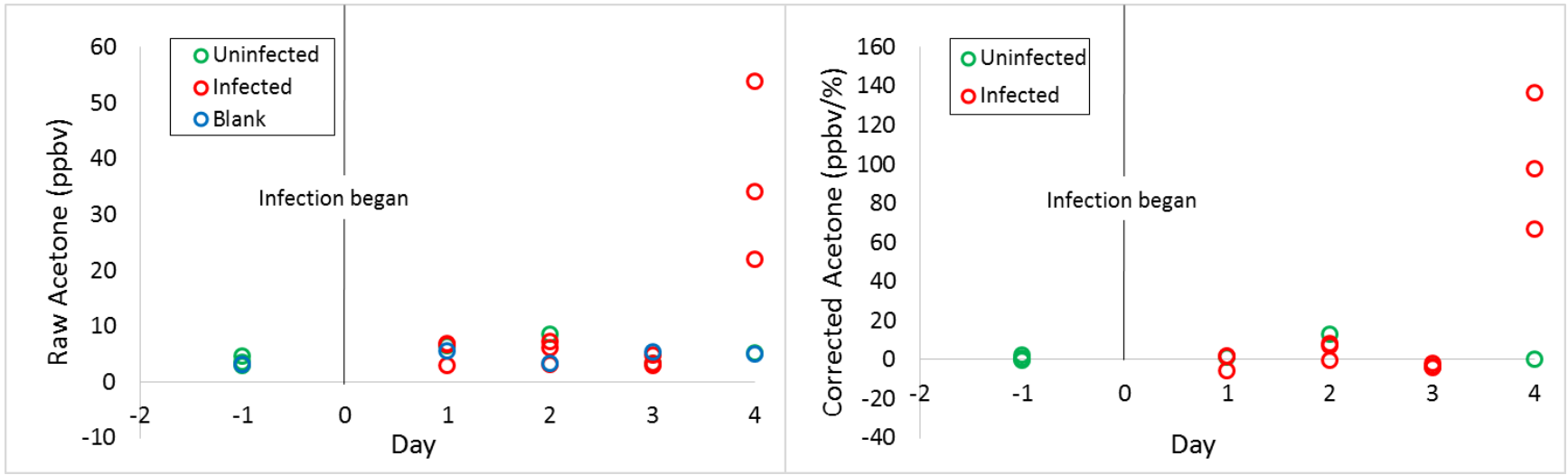

Figure 3.1. b. Graphs of raw and corrected data for acetone.

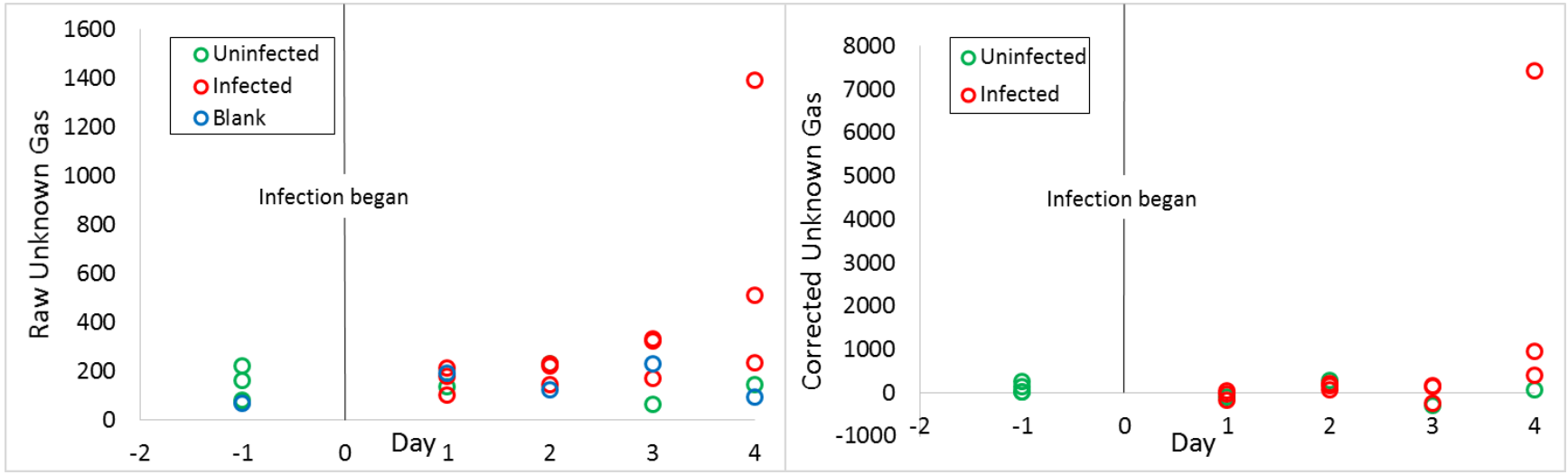

Figure 3.1. c. Graphs of raw and corrected data for unknown gas.

Discussion: Breath samples

The fact that carbon monoxide did not show a significant increase during the infection was consistent with the heme oxygenase-1 levels found in the blood of the mice. This enzyme would be a source of carbon monoxide in the bloodstream which would be eliminated from the body via the lungs. Acetone is a product of lipolysis and is one of the most abundant gases in human breath [7]. Additionally, isopropanol has been proposed as a metabolite of acetone in both humans and rats [8, 9]. This would suggest that the increase in the acetone concentration in the breath samples of the infected mice could be the cause of the increased isopropanol concentrations. Bacteria, such as *salmonella*, are known to ferment carbohydrates to alcohols which include acetone and isopropanol [10]. The accumulation of these alcohols in the intestines of the mice could lead to absorption by the intestinal lining into the blood stream. The presence of these alcohols in the blood stream could then be reflected in the exhaled breath, suggesting a possible pathway for these two gases. Unfortunately the identity of the third gas is not known at this time and therefore an explanation as to its presence is not possible.

Results: NMHC analysis for headspace samples.

As with the breath samples, the feces were analyzed using the Rowland Blake NMHC system to determine concentrations of a variety of gases. For this study, t-tests were performed to determine what gases may be considered different. However, the small sample size in this study meant that the t-test would not be reliable and was therefore only used as a preliminary investigation tool.

The results of the feces study will be presented in two ways, the first being in the raw form, meaning the blank concentrations were not subtracted and the data were not

corrected for weight differences. Additionally the corrected values were used, in which case the corresponding blank concentrations were subtracted and the resulting concentrations were divided by the weight of the feces sample in grams to normalize all samples. A t-test was then performed on both the raw and corrected data and gases that indicated a significant difference were selected. The results of the t-test for both the raw and corrected data are shown in Tables 3.5 a and b.

Table 3.5. a. Results of t-test for raw data showing interesting gases (WT=Wild type, KO=Knock-out).

	Raw	OCS	DMDS	CS_2	2-Heptanone	MEK	Heptanal	i-Propylbenzene
1 hour	ttest WT	0.12	0.18	0.28	0.13	0.38	0.42	0.13
	ttest KO	0.26	0.20	0.36	0.12	0.07	0.08	0.18
24 hour	ttest WT	0.44	0.24	0.14	0.48	0.00	0.10	0.24
	ttest KO	0.04	0.12	0.10	0.27	0.10	0.02	0.01
		Methylisobutylketone	n-Pentane	Isoprene	Acetaldehyde	3-Pentanone	Butanone	Acetone
1 hour	ttest WT	0.44	0.40	0.31	0.44	0.44	0.46	0.10
	ttest KO	0.18	0.34	0.38	0.38	0.13	0.13	0.12
24 hour	ttest WT	0.15	0.33	0.09	0.09	0.05	0.00	0.12
	ttest KO	0.29	0.40	0.05	0.35	0.18	0.48	0.37

Table 3.5. b. Results of t-test for corrected data showing interesting gases.

	Corrected	OCS	DMDS	CS_2	2-Heptanone	MEK	Heptanal	i-Propylbenzene
1 hour	ttest WT	0.05	0.38	0.21	0.10	0.03	0.06	0.43
	ttest KO	0.14	0.39	0.14	0.09	0.003	0.01	0.04
24 hour	ttest WT	0.28	0.20	0.12	0.01	0.01	0.01	0.02
	ttest KO	0.001	0.08	0.05	0.11	0.03	0.04	0.003
		Methylisobutylketone	n-Pentane	Isoprene	Acetaldehyde	3-Pentanone	Butanone	Acetone
1 hour	ttest WT	0.13	0.27	0.06	0.04	0.03	0.02	0.10
	ttest KO	0.003	0.35	0.13	0.03	0.003	0.01	0.11
24 hour	ttest WT	0.03	0.04	0.05	0.31	0.42	0.01	0.11
	ttest KO	0.19	0.01	0.01	0.09	0.06	0.04	0.04

In addition to the t-test, the interesting gases corrected results were analyzed to further determine the extent of the differences by taking the averages of the wild type (WT) uninfected, WT infected, and knock-out (KO) infected, for both 1 hour and 24 hour samples. The differences, delta values, for the WT uninfected vs. WT infected, and delta values for the WT uninfected vs. KO infected were then determined. The results of this analysis are shown in Table 3.6.

Table 3.6. Averages and delta values for corrected data showing interesting gases identified in t-test.

	Corrected	OCS (ppbv/g)	DMDS (ppbv/g)	CS2 (pptv/g)	2-Heptanone (ppbv/g)	MEK (ppbv/g)
1 Hour	Avg WT uninf	0.7	11	0.6	1	1
	Avg WT Inf	-0.9	7	2.4	6	28
	Avg KO inf	2.0	17	4.5	4	22
	Delta WT	**-1.7**	**-4**	**1.8**	**5**	**27**
	Delta KO	**1.2**	**5**	**3.9**	**3**	**21**
24 Hour	Avg WT uninf	1.3	2	3.4	6	18
	Avg WT Inf	13.6	13	202.4	82	52
	Avg KO inf	37.4	1267	279.8	78	74
	Delta WT	**12.3**	**11**	**198.9**	**75**	**34**
	Delta KO	**36.1**	**1265**	**276.4**	**72**	**55**
		Methylisobutylketone (pptv/g)	**n-Pentane (pptv/g)**	**Isoprene (pptv/g)**	**Acetaldehyde (ppbv/g)**	**3-Pentanone (pptv/g)**
1 Hour	Avg WT uninf	9	77	50	10	95
	Avg WT Inf	144	419	191	151	1359
	Avg KO inf	35	141	155	73	2340
	Delta WT	**135**	**341**	**141**	**142**	**1264**
	Delta KO	**26**	**64**	**104**	**64**	**2245**
24 Hour	Avg WT uninf	**108**	**284**	**76**	204	**203**
	Avg WT Inf	728	4726	3428	123	11
	Avg KO inf	1661	2198	4620	2162	3545
	Delta WT	**620**	**4442**	**3353**	**-81**	**-192**
	Delta KO	**1554**	**1915**	**4544**	**1958**	**3342**

Table 3.6. (continued). Averages and delta values for corrected data showing interesting gases identified in t-test.

		Heptanal (ppbv/g)	i-Propylbenzene (pptv/g)	Butanone (ppbv/g)	Acetone (ppbv/g)
1 Hour	Avg WT uninf	0.3	7	4	2
	Avg WT Inf	3.6	14	68	834
	Avg KO inf	3.8	24	53	95
	Delta WT	**3.3**	**8**	**64**	**832**
	Delta KO	**3.6**	**18**	**48**	**93**
24 Hour	Avg WT uninf	0.1	**19**	**38**	**49**
	Avg WT Inf	2.6	370	123	1988
	Avg KO inf	3.3	392	259	275
	Delta WT	**2.5**	**351**	**84**	**1939**
	Delta KO	**3.2**	**373**	**220**	**226**

Based on the information from the Tables, graphs were made to visualize differences for the interesting gases. The resulting graphs were visually analyzed to determine if differences existed between the uninfected and infected samples. To avoid errors that might have occurred due to the weight differences, only those graphs that showed a difference both in the raw and corrected data will be shown. These graphs are pictured in Figures 3.2. a, b, and c.

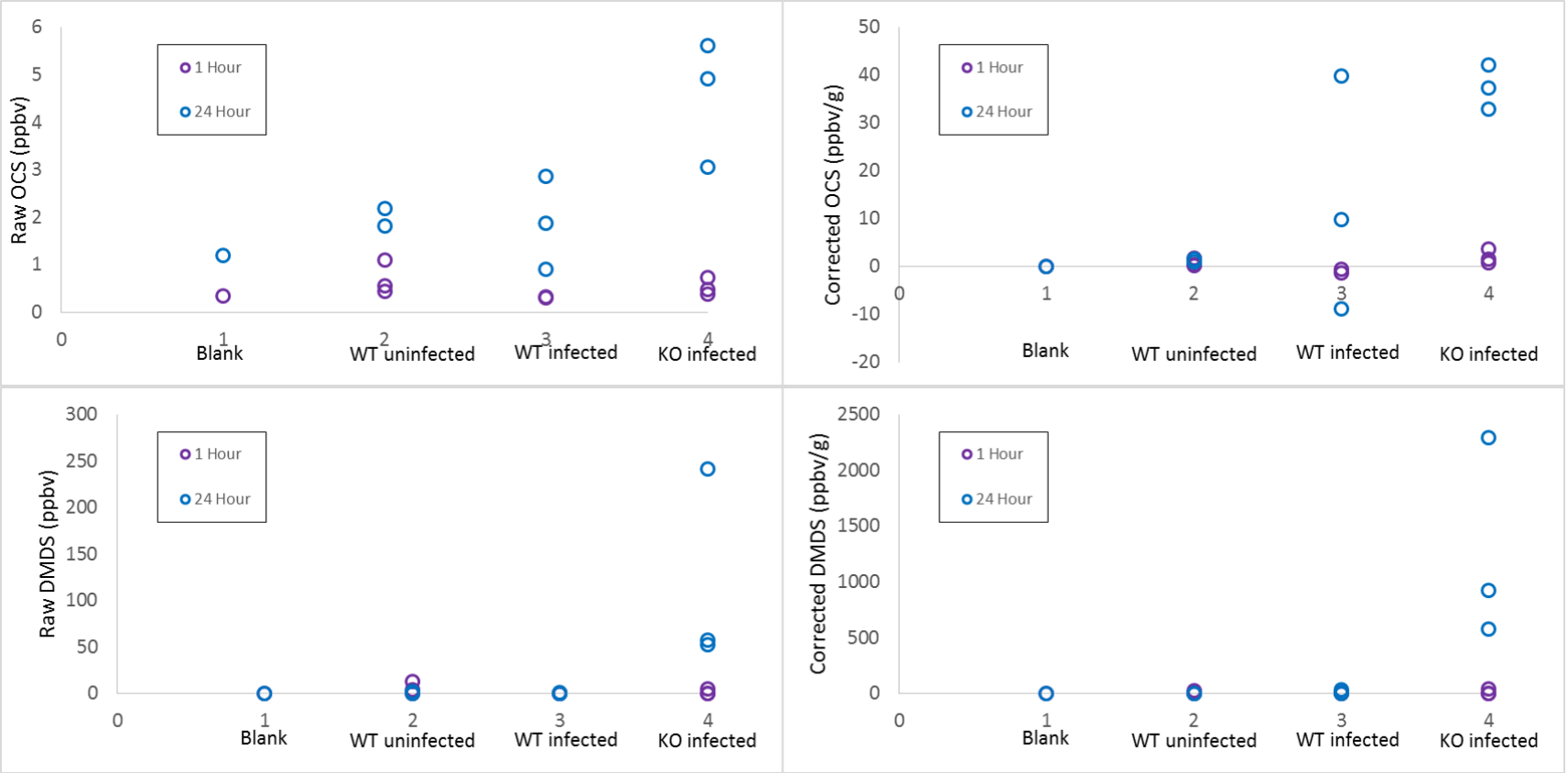

Figure 3.2. a. Graphs of gases identified as showing a difference between uninfected and infected samples for both raw and corrected data.

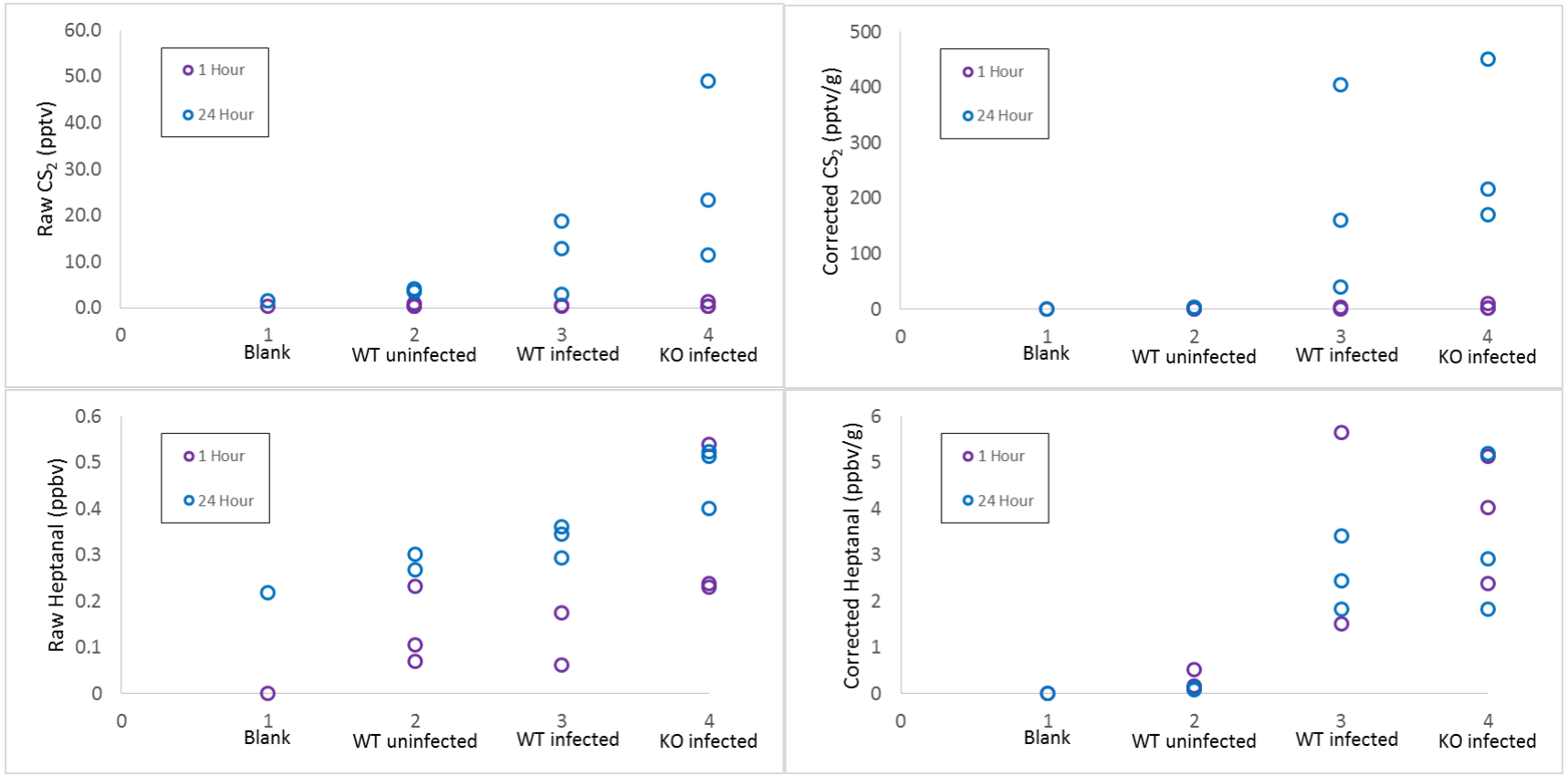

Figure 3.2. b. Graphs of gases identified as showing a difference between uninfected and infected samples for both raw and corrected data.

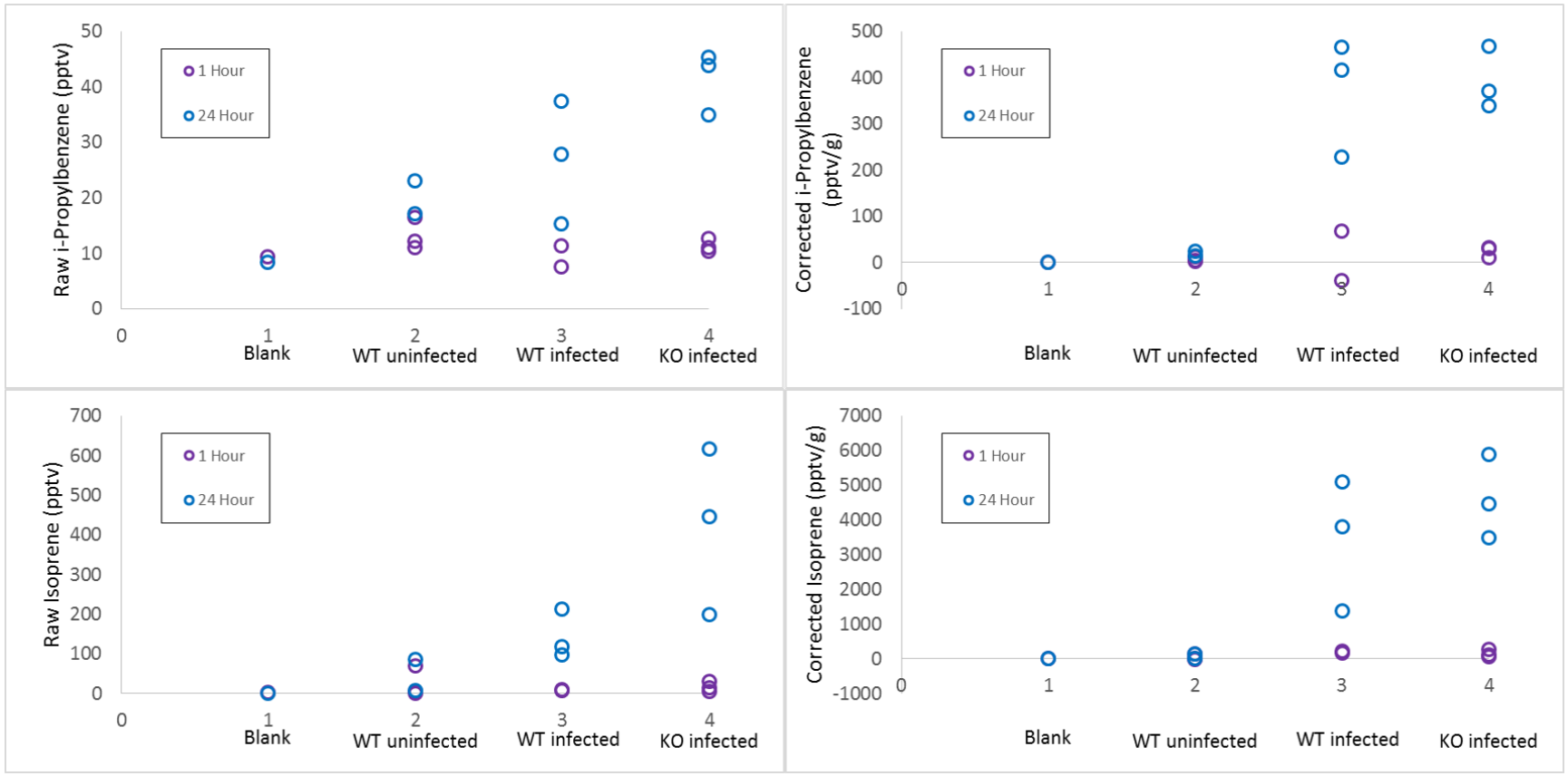

Figure 3.2. c. Graphs of gases identified as showing a difference between uninfected and infected samples for both raw and corrected data.

After the various forms of analysis, 6 gases were identified as being possible markers for *salmonella* infection in feces; Carbonyl sulfide (OCS), Dimethyldisulfide (DMDS), Carbon disulfide (CS_2), Heptanal, i-Propylbenzene, and Isoprene. To summarize the data from the Tables above, the delta values for these identified gases are shown in Table 3.7.

Table 3.7. Summary of delta values for gases identified as different.

Corrected	Delta WT (1 hour)	Delta KO (1 hour)	Delta WT (24 Hour)	Delta KO (24 Hour)
OCS (ppbv/g)	-1.7*	1.2	12.3	36*
DMDS (ppbv/g)**	-4.0	5.1	11	1265
CS_2 (pptv/g)	1.8	3.9	199	276*
Heptanal (ppbv/g)	3.3	3.6*	2.5*	3.2*
i-Propylbenzene (pptv/g)	7.8	18*	351*	373*
Isoprene (pptv/g)	141	104	3353*	4544*

*Indicates t-test value was significant for corrected data for this parameter.
**No significant t-test value was observed for DMDS however it was included due to the large delta value for 24 hour KO and visual difference observed on graph.

Discussion: Feces headspace samples.

Several issues exist with this data set, the main issues being the small sample size and lack of a repeat study. The data presented here however, can serve as a starting point for further studies that may be performed. If future studies are carried out, a larger sample size would be highly recommended. Additionally, the presence of a control sample collected from uninfected knock-out mice would also likely enhance the statistical analysis of the resulting data, rather than having to compare the uninfected wild type with the infected knock-out samples. Finally, a repeat study would also benefit from normalization of the weight of samples prior to incubation and collection of the headspace. This would control for differences in emitted gases based on weight without the need to divide the resulting concentrations as done with this data set.

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Chapter 4: *Borrelia Hermsii*

Introduction

Borrelia hermsii is a bacteria in the spirochete family which causes relapsing fever in humans [1]. This bacteria is transmitted to humans via tick bite and is a relative of the tick borne *Borrelia burgdorferi* [2, 3]. *Borrelia burgodoferi* infection causes the disease referred to as Lyme disease [3]. In the United States, this disease is the most common of all vector borne diseases [4]. In 2012 the CDC reported 30,000 cases of Lyme disease in the United States alone and estimated that the actual number of cases could be much higher than reported [5]. Currently, Lyme disease diagnosis is not standardized leading to under and over diagnosis of the disease [6]. Common methods of diagnosis include blood tests, however the bacteria are only detected in the blood of around 45% of American patients and less than 8% of European patients in the first few weeks of the disease [6]. For this reason, alternative diagnostic means are highly sought after for Lyme disease. Because of its lower bio-safety hazard level and relationship to *Borrelia burgdorferi*, *Borrelia hermsii* was investigated for diagnostic potential in this thesis.

Methods

For all *Borrelia hermsii* studies the breath tower was used with individual mice connected for sampling. The first of the studies was performed using 20 normal Balb-C mice from Dr. Alan Barbour's lab. Of the 20 mice, five were uninfected while the remaining 15 were infected prior to the start of sampling. Breath samples were collected once a week for three weeks with a blank sample collected at the start of each sampling day. For this study, the tower clean air flow was set at 1 L/min for both the flush and sample portions

with a six minute flush and two minute sample collection. This sample set will be referred to herein as Borrelia Balb-C #1.

The second study was performed again using 20 mice however this time severe combined immunodeficient mice (SCID) were obtained that would be unable to clear the infection on their own during the sampling period. Also for this study, all mice were uninfected at the beginning of the study with two days of background sampling prior to infection. On the third day, 16 of the 20 mice were infected with the remaining four mice serving as controls being uninfected for the remainder of the study. Sampling resumed on day five (infection day 2) and continued for all 20 mice through day nine. After sampling on day nine, the mice were given antibiotics which continued twice a day for days 10-12. For the treatment portion of the study only 10 mice were sampled with two uninfected and eight infected (treated). For this study, the tower clean air flow was set at 1 L/min for both the six minute flush and two minute sample collection. This sample set will be referred to herein as Borrelia SCID #1.

After the first two studies it was noted that there were several samples with low CO_2 values which were attributed to two possible factors. The first was the flow rate of the background during sampling which would lead to dilution of the breath sample. During the third study, which used Balb-C mice, the flow was reduced during the sampling portion only to 0.5 L/min to reduce the effect of dilution on the breath sample. The flush rate was unchanged to avoid greatly increasing the amount of time the mice were subjected to the tower so as not to stress the mice. Additionally it was determined that the plungers attached to the chambers which housed the mice were too short as manufactured and were not effective at holding the mice in place during sampling. This led to mice being able to

turn during the sample collection which again enhanced the effect of the dilution of some breath samples. This was corrected during the third study by replacing the screw attached to the plunger with one that was significantly longer, allowing better restraint of the mice during sampling. The third study was conducted on four Balb-C mice, with a blank sample collected each day for a total of four days. The mice were all uninfected for the first two sampling days after which all four were infected. This sample set will be referred to herein as Borrelia Balb-C #2

The fourth and final Borrelia study was performed using 18 SCID mice with three days of background sampling on which all mice were uninfected. After sample collection on the third day, 14 mice were infected with four remaining uninfected for the duration of the study. On day five, day two of infection, sampling resumed and continued through day eight. At the end of sampling on day eight, all uninfected and five infected mice were given antibiotic treatment. By day nine, four of the infected mice had succumbed to the infection leaving 10 infected mice of which five had been given antibiotics. These 10 mice along with the four uninfected mice were sampled on day 9. On day 10, the second day of treatment, only the mice that had been treated remained leaving the four uninfected and five infected (treated) mice for sampling. Breath samples were also collected on days 11-13 to determine the effect of treatment over time. The flow for this study was set at 1 L/min for the flush and reduced to 0.5 L/min during sample collection. The flush was conducted for six minutes with a four minute sample collection. This sample set will be referred to herein as Borrelia SCID #2.

Results

For all studies, the use of individual mice in the tower led to a relatively small sample of breath collected. This led to issues when analyzing the samples for most VOCs because of the negligible concentrations of gases when compared to the blank samples. This can best be demonstrated by comparing the individual sample chromatogram to one collected from a study that used groups of mice, such as the salmonella study that was discussed in the previous chapter. Figure 4.1 shows the negligible difference in the chromatogram of an individual mouse sample when compared to the blank, and the enhanced concentration that is obtained when groups were used.

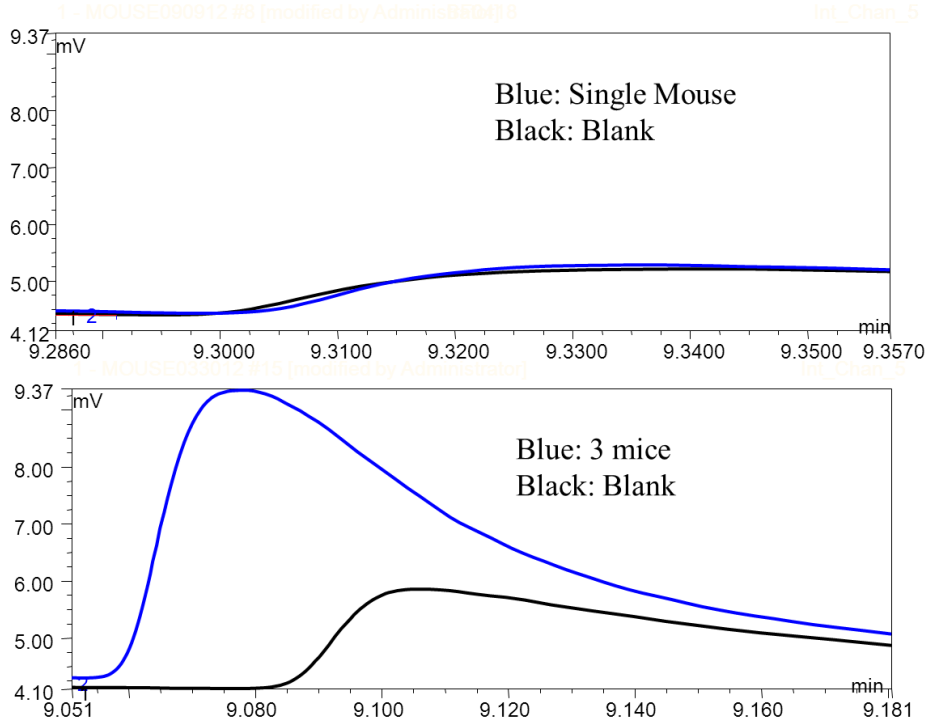

Figure 4.1. Individual mouse compared to blank chromatogram for gas on the NMHC system (top) and group of three mice compared to blank for the same gas on NMHC system.

Despite the small sample of breath collected, there was sufficient concentrations of both CO and CO_2 for a difference to be observed between the breath and blank samples.

This was especially true of the fourth study (Borrelia SCID #2) after the sampling procedure had been optimized. Due to the fact that the Balb-C mice were able to fight and clear the infection during the sampling period, little to no effect was seen in the breath of these mice throughout the sampling for Borrelia Balb-C #1 which was conducted only once a week. In addition, the Borrelia Balb-C #2 study was primarily conducted to optimize sample conditions and therefore the results were inconclusive. The results of the SCID studies however did show a difference between the infected and uninfected samples throughout the sample period and will be discussed further.

In SCID #1, the sample procedure had not yet been optimized leading to lower concentrations of CO_2 than those seen in the second SCID study. For example, the average corrected CO_2 concentration for the uninfected mice in the SCID #1 study was only 0.12% compared to an average of 0.36% for the uninfected mice in the SCID #2 study. Additionally, there were issues with the mice turning around in the restraints during sampling in the SCID #1 study which was reflected in the CO_2 concentrations as well. For this study, the blank concentration averaged 0.05%. Therefore samples with less than twice the blank concentration, or 0.10%, were determined not to be representative of a good breath sample and were discarded. Once the data had been analyzed, graphs were made to visualize any difference between the infected and uninfected samples over time. First, the raw CO concentrations were plotted by sample day followed by the corrected (blank subtracted) by sample day. These graphs are shown in Figures 4.2. a and b.

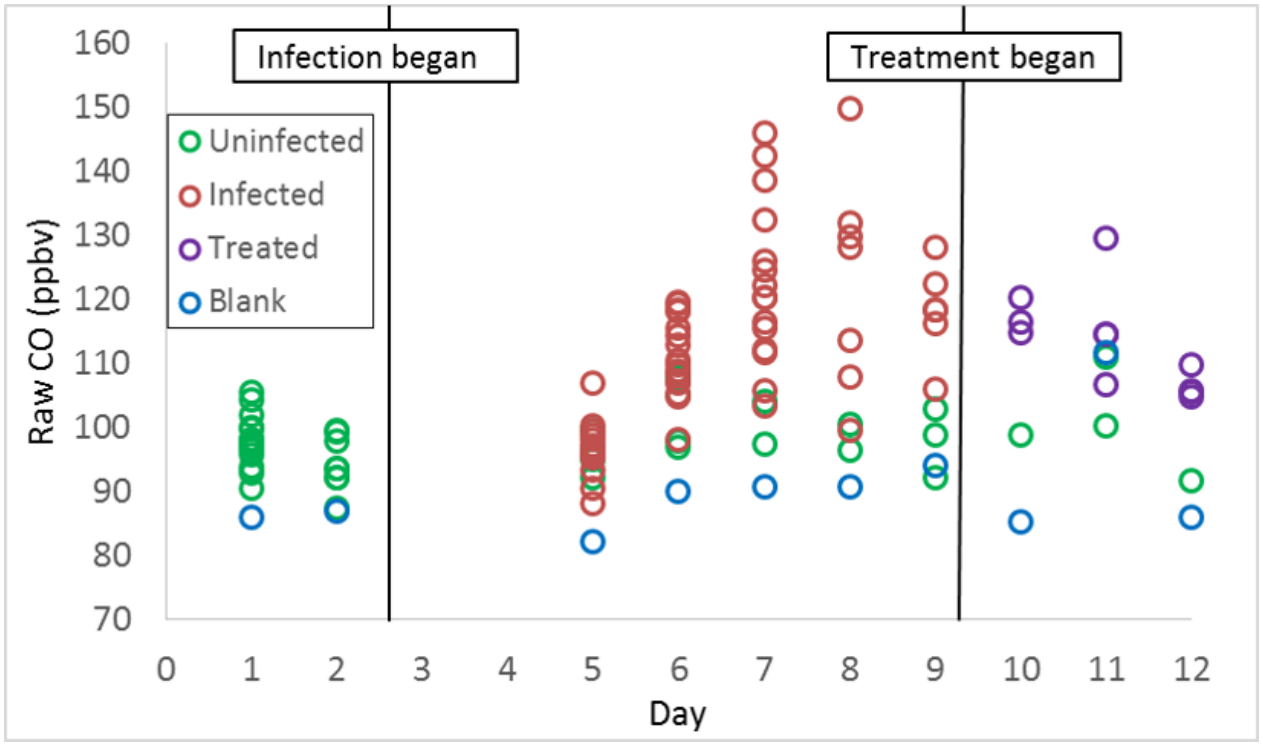

Figure 4.2.a. Raw CO concentration plotted over time for SCID #1 study separated by blank, uninfected, infected, and treated samples.

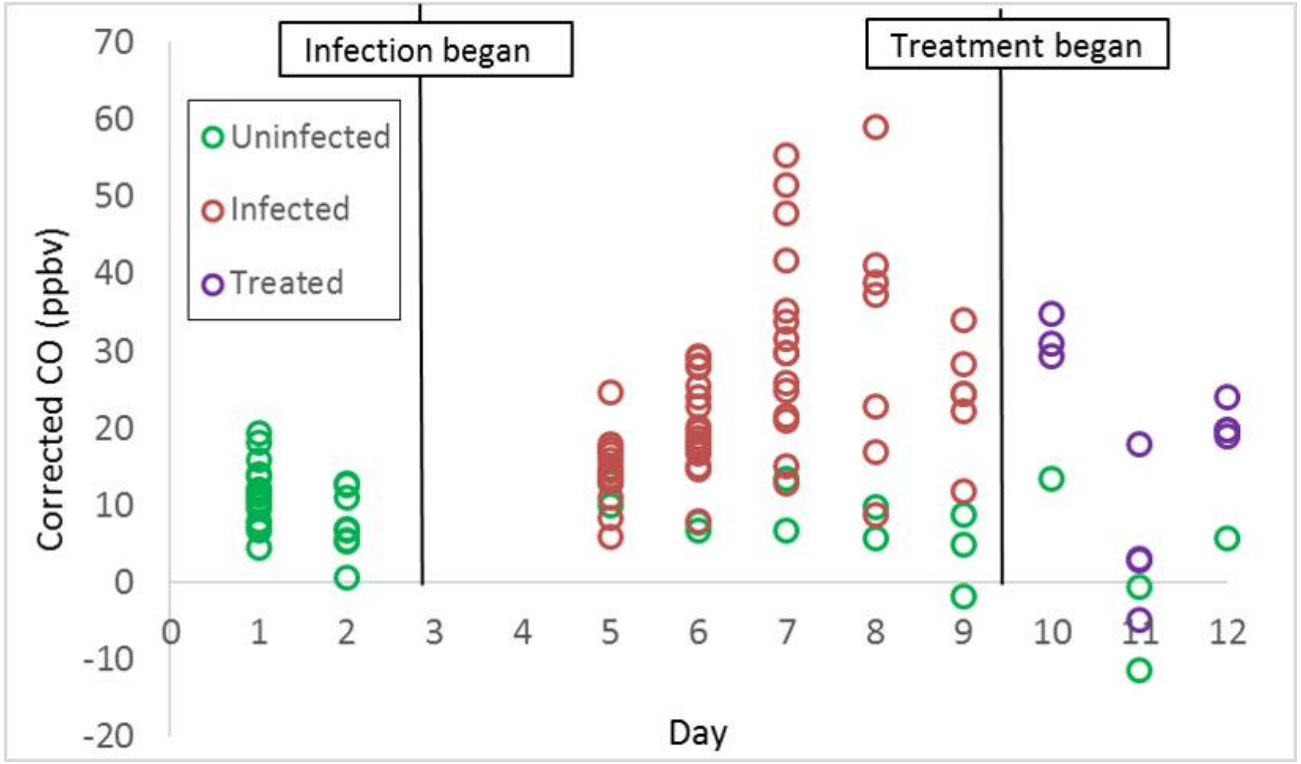

Figure 4.2. b. CO concentration after subtraction of blank concentration plotted over time for uninfected, infected, and treated samples.

The data used to make the graphs above were also analyzed by performing t-tests. For the raw CO concentrations, there was a significant difference between all uninfected and infected samples over the study period. The p-value for this t-test was found to be $<10^{-4}$. A t-test performed on the uninfected samples for the entire study period vs. the infected samples on days seven and eight was also found to be significant with a p-value of $<10^{-4}$. Averages for CO were also taken for all samples and were found to be 98 ppbv (87-111) for the uninfected samples and 113 ppbv (88-150) for the infected samples. For days seven and eight, the average for the infected samples was 123 ppbv (100-150).

These t-tests were also performed on the corrected CO concentrations which were determined to be significant as well. The p-value for all uninfected vs. infected samples for the corrected data was $<10^{-4}$, and the p-value for all uninfected vs. infected samples on days seven and eight was also $<10^{-4}$. Averages for the corrected CO concentrations were 9 ppbv (-11-19) for the uninfected samples and 24 ppbv (6-59) for the infected samples. On days seven and eight, the average for the infected samples was 32 ppbv (9-59). In order to account for differences in breathing rates between the mice, the corrected CO concentrations were divided by the corrected CO_2 concentrations. The results were plotted over time for the uninfected, infected, and treated samples and shown in Figure 4.3.

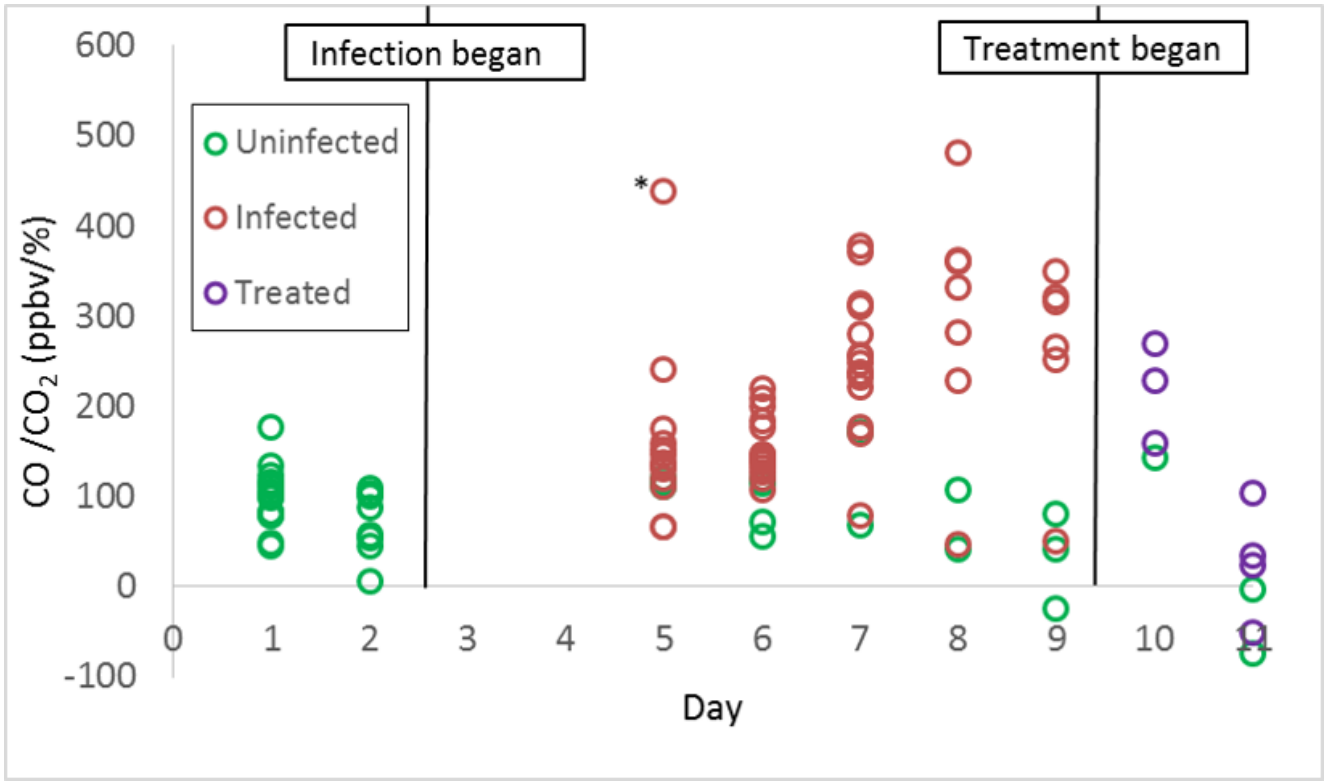

Figure 4.3. Corrected CO concentrations divided by corrected CO_2 concentrations plotted over time for uninfected, infected and treated samples. *This mouse had the highest CO (25 ppbv) and lowest CO_2 (0.056%) of day 5.

Due to an unusually high concentration of CO_2 in the blank sample on day 12 (0.175%), the data for this day were not included. The p-value determined from the t-test on all uninfected vs. infected samples for the corrected CO/CO_2 concentrations was also found to be significant at $<10^{-4}$. For the uninfected samples vs. the infected samples on day seven and eight, the p-value was found to be $<10^{-4}$. The uninfected average was 84 ppbv/% (-75-176) for the corrected CO/CO_2 concentrations. For the infected samples, the average was 210 ppbv/% (46-481). On days seven and eight, the infected average was 266 ppbv/% (46-481). Due to the fact that the concentrations seemed to be declining on day nine, average CO/CO_2 of 259 with maximum of 350, no treatment effect could be determined.

For the SCID #2 study, plots were made of the raw CO concentrations over time for the blank, uninfected, infected, and treated samples as well. As with SCID #1 the corrected CO concentrations, after subtraction of the blank, were also plotted for the uninfected, infected, and treated samples. Upon completion of the analysis, it was determined that two of the mice that had been injected with the *Borrelia hermsii* bacteria did not in fact become infected and were therefore plotted with the uninfected samples. In addition, the samples collected the day after infection were not significantly different than the uninfected samples, so they were plotted as uninfected as well. The resulting graphs are shown in Figures 4.4. a and b.

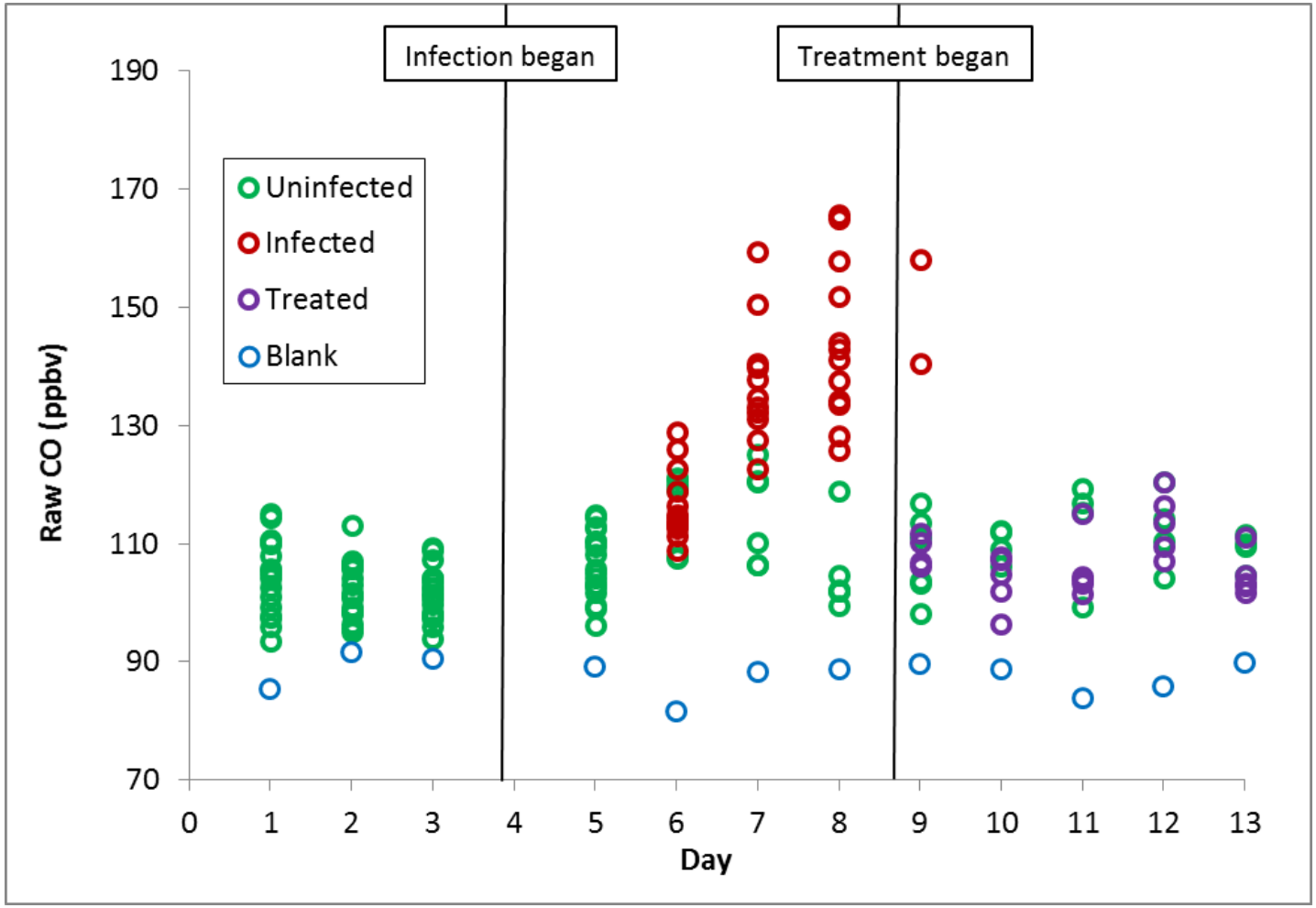

Figure 4.4. a. Raw CO concentration plotted over time for SCID #2 blank, uninfected, infected, and treated samples.

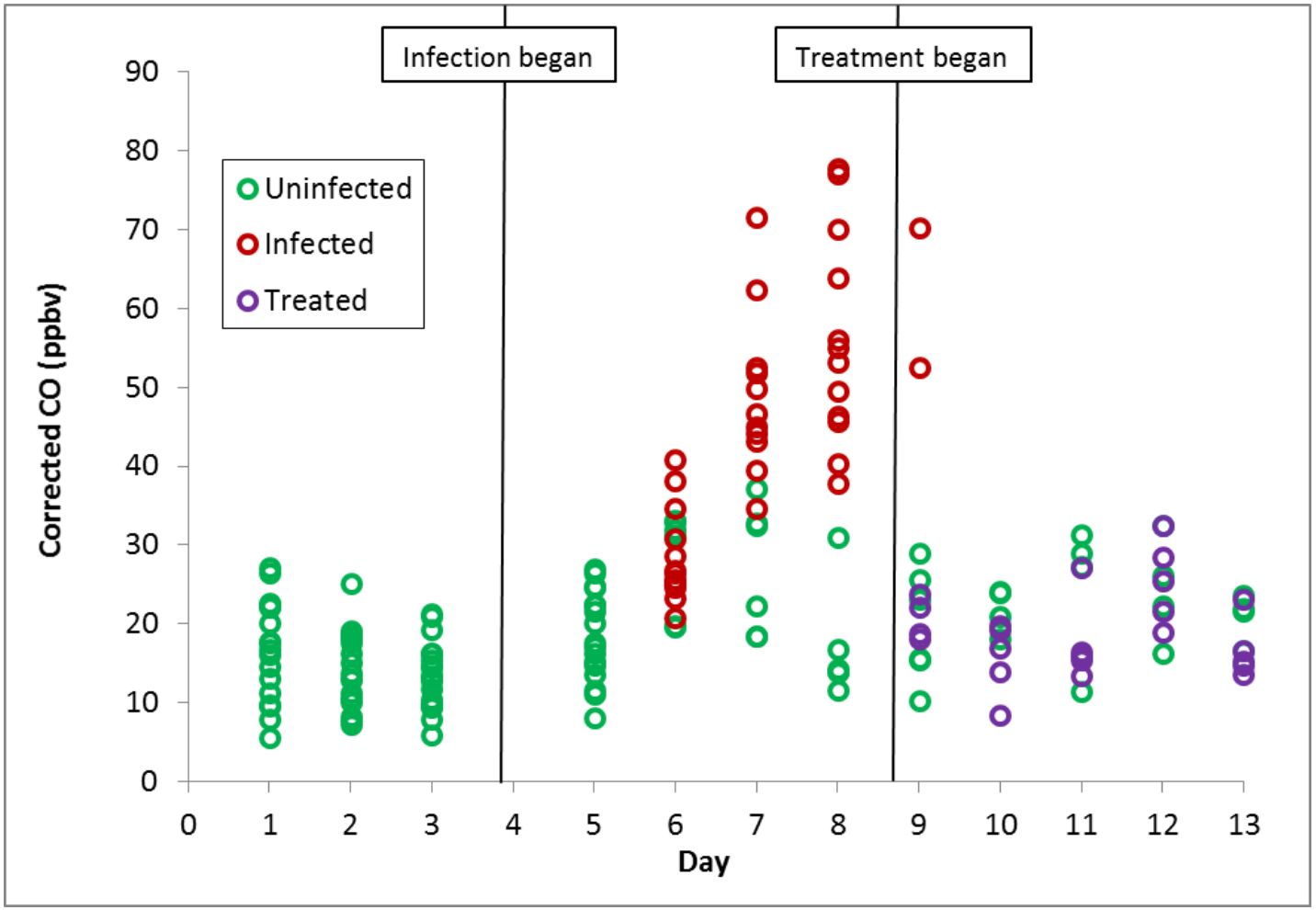

Figure 4.4. b. Corrected CO concentration after subtraction of blank plotted over time for uninfected, infected, and treated samples.

For the raw CO concentrations, the p-value for the uninfected samples vs. the infected samples for the whole study period was found to be $<10^{-4}$ suggesting a significant difference in these samples. For the infected vs. treated samples, the p-value was also significant at $<10^{-4}$. However for the uninfected vs. treated samples, the p-value was not significant at 0.56. This suggests that there was a treatment effect observed in this study with the raw CO concentrations. The average raw CO concentrations were 106 ppbv (94-125) for the uninfected, 133 ppbv (109-166) for the infected, and 107 ppbv (96-121) for the treated samples.

The p-value for the corrected CO concentrations determined for the uninfected vs. infected samples was $<10^{-4}$. For the infected vs. treated samples the p-value was $<10^{-4}$ and for the uninfected vs. treated samples it was 0.18. The average corrected CO concentration for the uninfected samples was 18 ppbv (6-37), for the infected samples the average was 42 ppbv (21-78), and for the treated samples it was 15 ppbv (8-33). Again in order to account for differences in breathing rates, the corrected CO concentrations were divided by the corrected CO_2 concentrations and plotted over time. The resulting graph is shown in Figure 4.5.

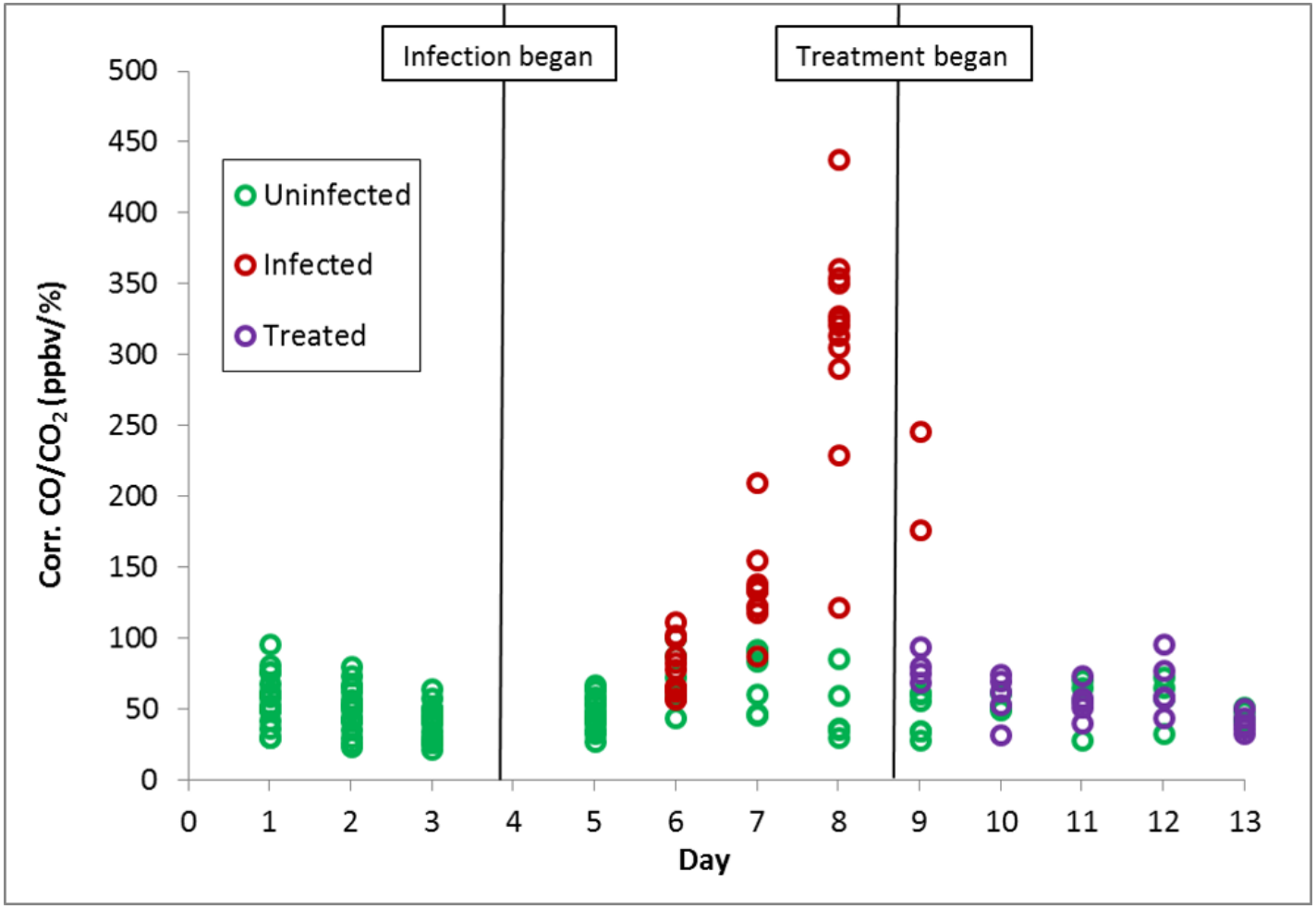

Figure 4.5. Corrected CO divided by corrected CO_2 and plotted over time for uninfected, infected, and treated samples.

For the corrected CO divided by CO_2 the uninfected and infected samples were found to be significantly different with a p-value of $<10^{-4}$. The p-value for the infected vs. treated samples was $<10^{-4}$. In the case of the corrected CO divided by CO_2 data, the uninfected and treated samples were determined to be significantly different with a p-value of $2.0*10^{-3}$. This could possibly be due to the fact that there was a wider range of values in the uninfected samples compared to the treated samples for this data set, while the range of the uninfected and treated samples were closer to each other for the raw and corrected CO values. As another way to visualize the difference between the CO and CO_2 concentrations for the uninfected and infected samples, the concentrations were plotted against one another for the uninfected data and for the infected data on day eight (height of infection) and least squares linear regression was performed. The resulting graph is shown in Figure 4.6.

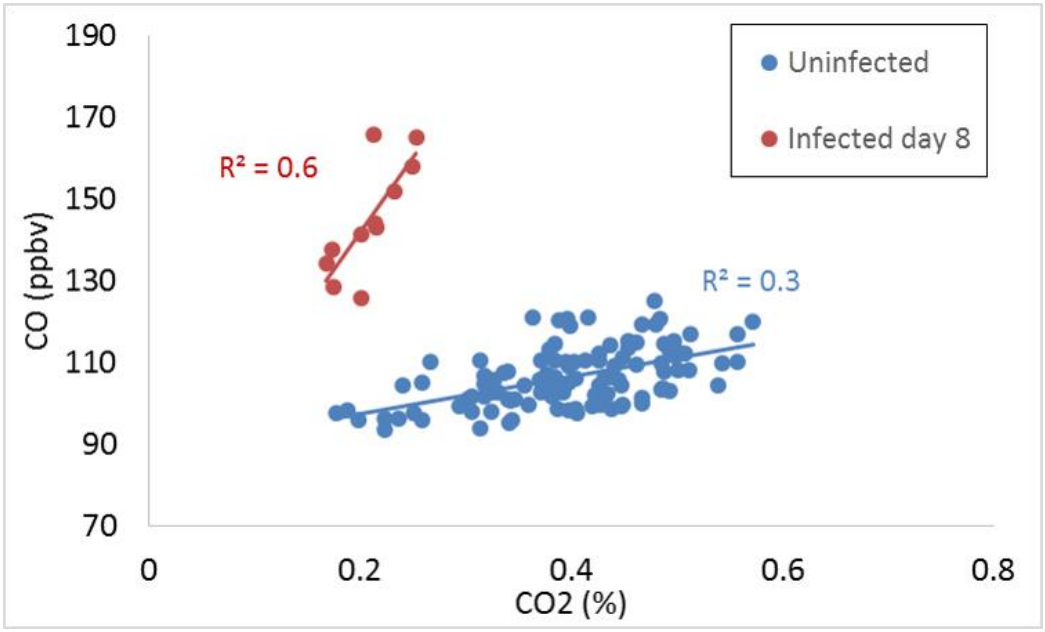

Figure 4.6. CO concentration plotted vs. CO_2 concentration for all uninfected data and infected data collected on day 8.

The results demonstrated that while the CO and CO_2 values correlated for both the uninfected and infected samples, they did so under different linear regressions. For example, the concentrations of CO for the infected samples were around 1.5 times higher than the uninfected samples at a given CO_2 concentration. This further validated the need to normalize CO concentrations relative to CO_2 for this study. In order to determine the effect of the infection/treatment on individual mice, the CO/CO_2 concentrations were plotted over time by mouse and labeled as uninfected, infected and treated accordingly. These graphs are shown in Figures 4.7. a, b, and c.

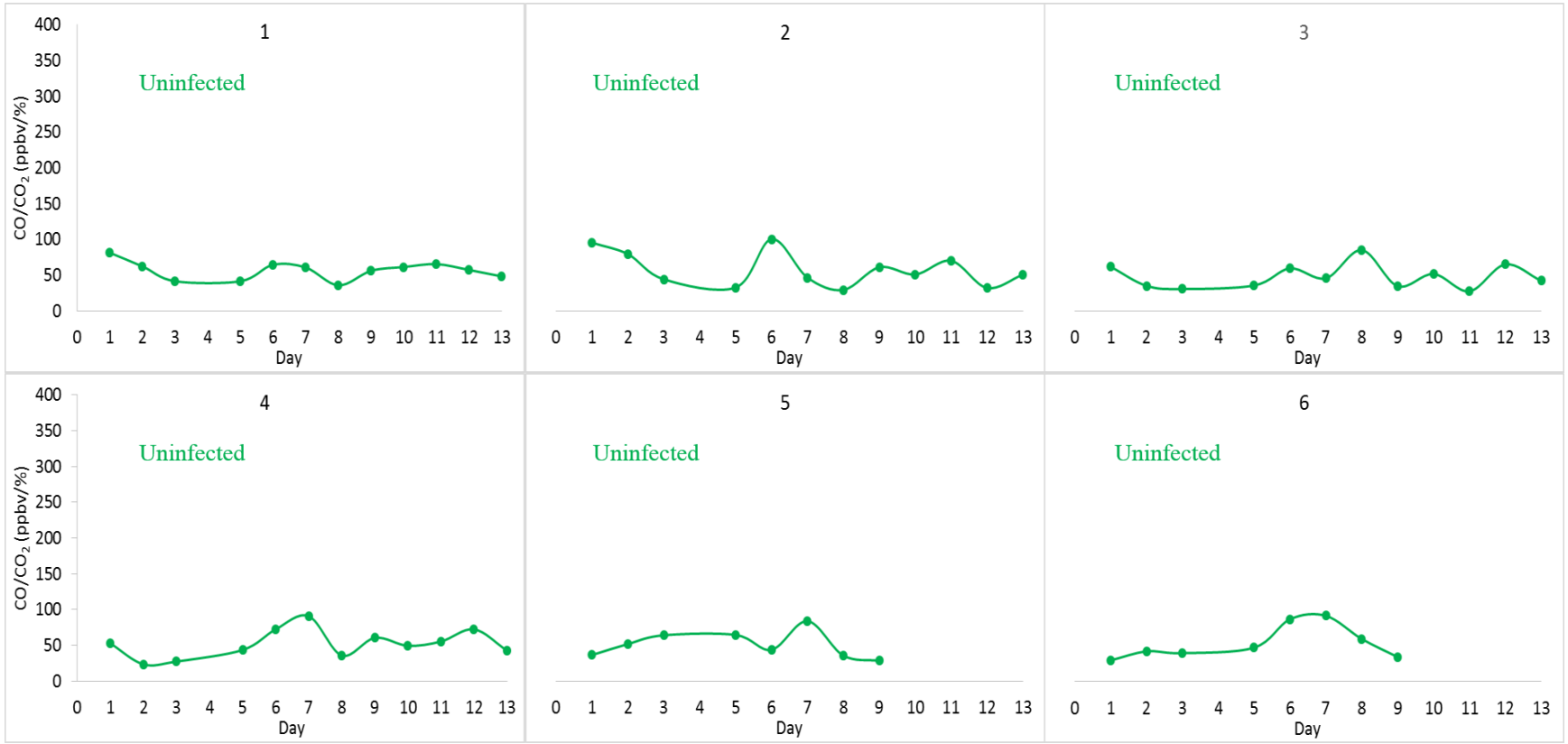

Figure 4.7. a. Individual plots of CO divided by CO_2 by mouse for uninfected samples.

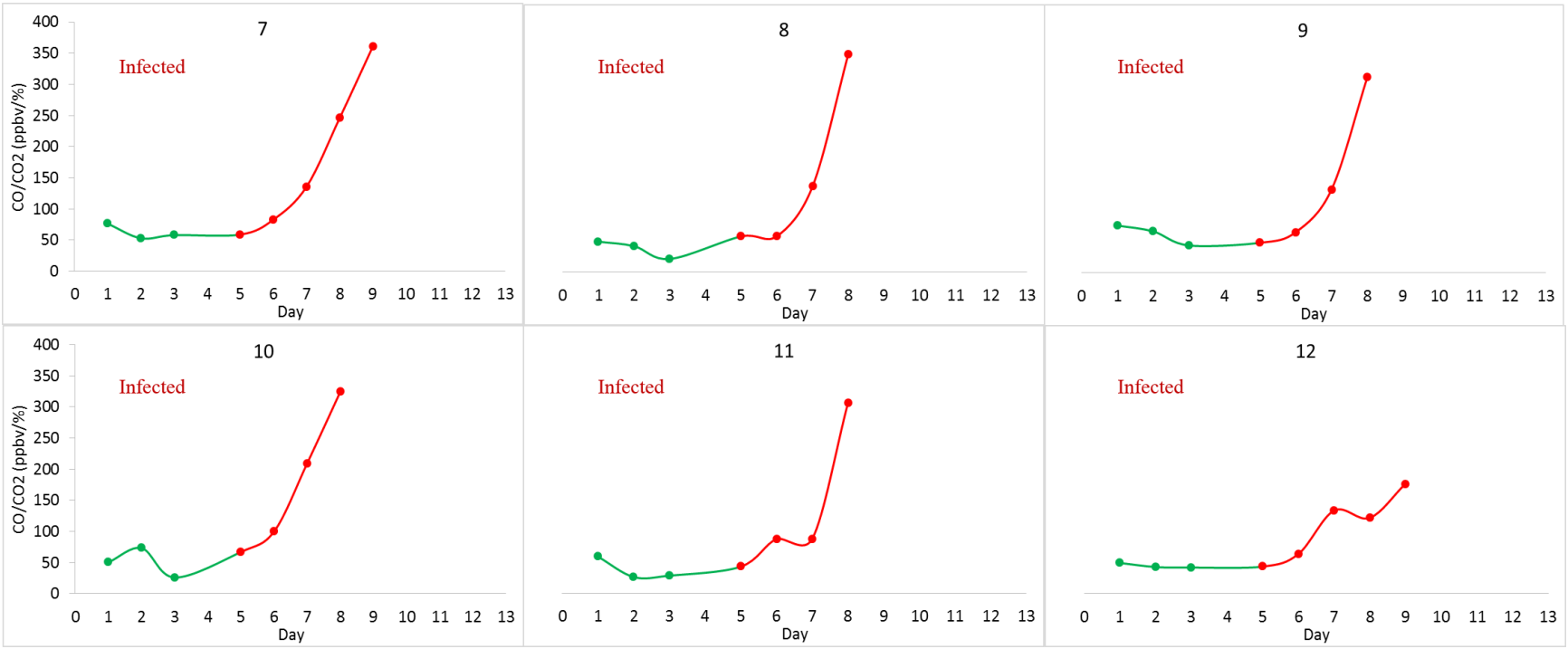

Figure 4.7. b. Individual plots of CO divided by CO_2 by mouse for infected samples.

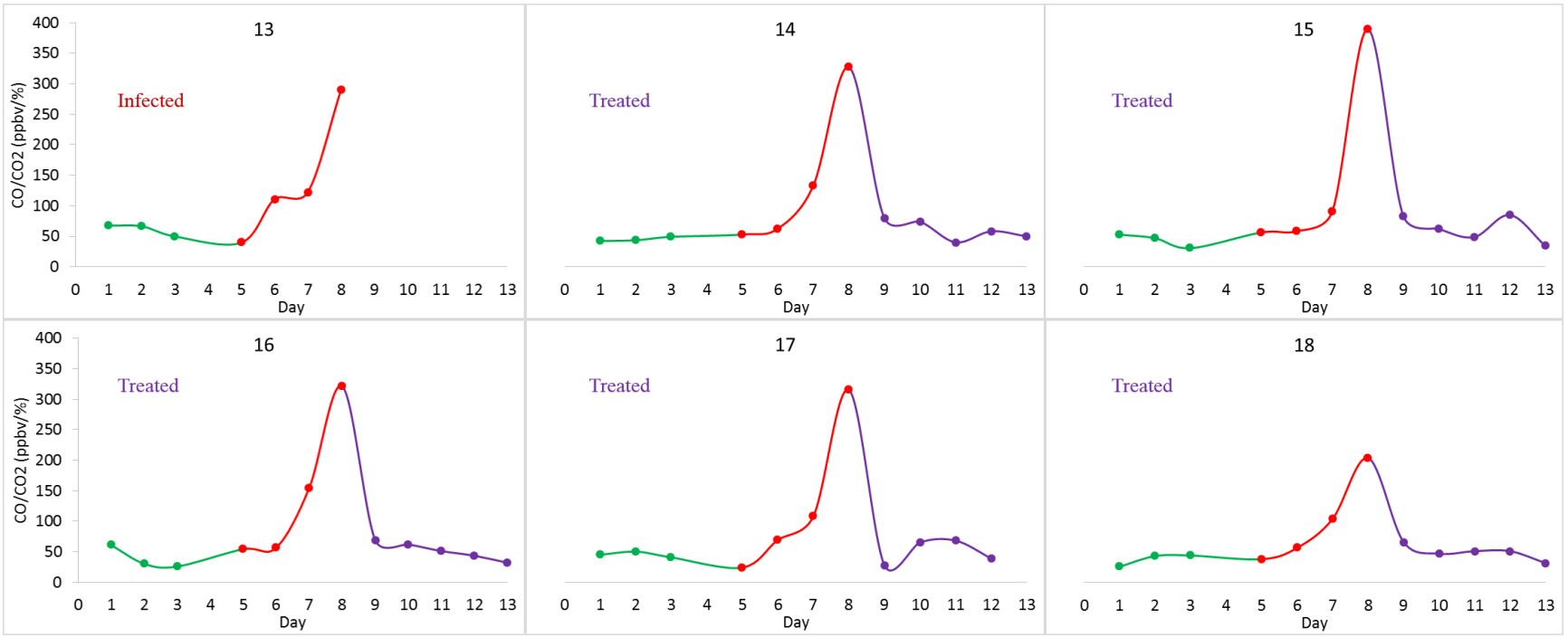

Figure 4.7. c. Individual plots of CO divided by CO_2 by mouse for infected sample and treated samples.

These graphs indicate that while the concentrations of CO divided by CO_2 varied little over the course of the experiment for the uninfected samples, the infected samples showed a significant rise in this concentration after infection. In addition, the treated samples show that the concentrations initially rose however fall to the initial uninfected range within less than a day of treatment. At the time of euthanasia of the mice that remained to the end of the study, serum samples were collected and analyzed by Dr. Barbour's lab. The results of the heme oxygenase-1 levels were plotted against the concentrations of CO/CO_2 to determine if any correlation was present. The resulting graph is shown in Figure 4.8.

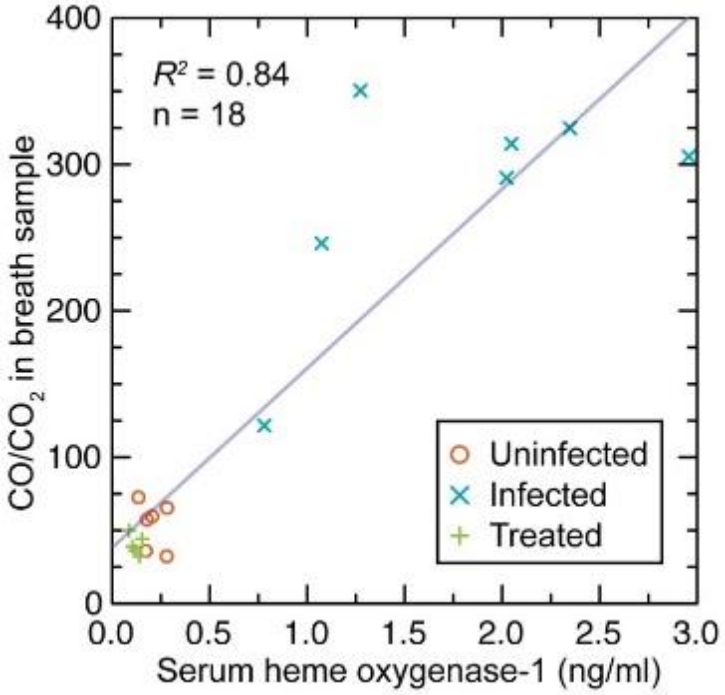

Figure 4.8. CO divided by CO_2 concentrations plotted vs. heme oxygenase-1 levels measured in serum of mice [7].

The results of this comparison showed a correlation between the CO/CO_2 levels in the breath of the uninfected, infected and treated mice with the levels of heme oxygenase-1

found in the serum samples. In fact it was determined that when the heme oxygenase-1 levels in the blood were ≤0.5 ng/mL the CO/CO_2 value was ≤100 (ppbv/%) and when the heme oxygenase-1 levels were >0.5 ng/mL the CO/CO_2 values were >100. The p-value for the heme oxygenase levels that were >0.5 ng/mL (infected samples) vs. those that were ≤0.5 ng/mL (uninfected and treated samples) was found to be significant at $<10^{-4}$.

Discussion

Unfortunately because of the small amount of breath sample collected when using individual mice an analysis of the samples for a variety of VOCs was not possible. However, the high concentrations of CO and CO_2 in breath allowed for a quantitative analysis of these gases in the breath samples. In both of the SCID studies, a significant increase in the CO as well as the CO/CO_2 concentrations of the infected mice was observed. While no treatment effect was observed in the first study, a significant effect was observed in the second SCID study. The fact that the levels of CO/CO_2 returned to those observed in the uninfected samples within 12 hours or less of treatment was very promising. This would suggest that not only could the concentrations of CO/CO_2 be used to diagnose this infection, and possibly a variety of other diseases, but also could be used as a treatment monitoring tool. Additionally, the correlation between the heme oxygenase-1 levels in the blood with the CO/CO_2 levels is also significant as CO and heme oxygenase expression have been noted to increase as a response to inflammation, sepsis, and oxidative stress in a variety of diseases including malaria [7].

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Chapter 5: Endotoxin

Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is a component in the cell wall of gram-negative bacteria, such as *E. Coli* [1]. Infection with bacteria containing LPS endotoxin causes severe inflammatory response which can lead to a condition of blood poisoning, referred to as sepsis, which is highly lethal [2]. The presence of the endotoxin in the cell wall of the bacteria leads to issues with treatment because while antibiotics will kill the bacteria, the rupture of the bacterial cells will lead to release of the endotoxin into the body, which can in turn lead to sepsis [3]. For this reason, alternative treatments that can treat the bacterial infection without the release of endotoxin are being investigated [4]. In order to effectively treat these infections, as well as the sepsis that can occur as a result, they must be able to be rapidly diagnosed and easily monitored. For this reason, the effect of infection of mice and rats with LPS endotoxin from *E. Coli* bacteria on breath gas signature was investigated. In addition, another component of bacterial cell walls, outer surface protein A (OSP A) from *Borrelia burgdoferi* was examined.

Methods: Tower samples

In the first endotoxin study, ten mice were used with background samples having been collected for two days prior to infection. On the third day, three of the mice were injected with 50 μg of endotoxin (low dose), four mice were injected with 250 μg of endotoxin (high dose), and three of the mice remained uninfected. Samples were collected at midday on the third day and again roughly six hours later. Samples were again collected twice a day (roughly 6 hours apart) on day four. On days five, six, and eight samples were collected once a day beginning in the morning. After sampling on day five, one of the mice

that had received the high dose expired. At the start of each set of sample collections, a blank sample was collected from an empty restraint tube attached to the tower. This study will be referred to as Endotoxin LPS #1.

For the second study, 14 mice were used with background samples being collected one day before infection. On the second day, 11 of the mice were injected with LPS endotoxin, six of which would be sensitive to the infection and five of which would be resistant. The three mice remaining were uninfected for the duration of the study. Samples were collected once a day for days two, and three with collection occurring in the evening on day three followed by the morning of day four. As in all other breath studies, blank samples were also collected at the start of each sample collection period. This study will be referred to as Endotoxin LPS #2.

A third study was conducted using ten mice with a single background collection prior to infection. On the second day, five of the mice were injected with 50 μg of OSP A from the Lyme disease causing *B. burgdorferi.* Samples were collected once a day on the evening of day two and the morning of day three, with blank samples collected at the beginning of each sampling period. This study will be referred to as Endotoxin OSP A.

In order to determine the effect of endotoxin on a variety of gases in the breath, a fourth study was conducted using groups of mice. For this study, groups of five mice each were attached to the tower at one time with a total of six groups. Background samples were collected on the morning of the first day with infection occurring in the afternoon of day two. For the infection period, three of the six groups of mice received injections of 10 μg LPS endotoxin per gram weight of mouse. Samples were collected on day two, four hours post injection and again on day three roughly 20 hours post injection. For the blank

samples in the group study, five empty chambers were attached to the tower. This study will be referred to as Endotoxin groups.

A final study using the breath tower was conducted using rats which would give a larger amount of breath volume per sample, while still allowing for individual sampling. This study was performed using four rats with background samples being collected once a day for six days prior to infection. On the seventh day, the rats were all injected with LPS endotoxin after which samples were collected four, seven, ten, and 24 hours after injection. Again, samples were collected from an empty restraint tube at the start of each sampling period. This study will be referred to as Endotoxin rats.

Methods: Bulb samples

The rats from the endotoxin tower study were also sampled using the glass bulbs described in the methods chapter of this thesis. Samples were collected from each of the four rats once a day for three days prior to infection. On the day of infection, two of the rats were sampled four hours post injection with the remaining two being sampled ten hours post injection. The results of the bulb study were compared to the results obtained from the tower.

Results: CO and CO_2

The samples for all studies were analyzed for CO and CO_2 beginning with Endotoxin LPS #1. In this study, the mice were separated into three groups, uninfected, low dose, and high dose, with the low and high dose mice receiving injections of 50 μg or 250 μg of LPS endotoxin, respectively. The results were subjected to t-tests and plotted in both the raw form of CO/CO_2 as well as the corrected CO/CO_2 (after subtraction of blank samples) as in previous studies. The resulting graphs showed an increase in the ratio of CO/CO_2 in both

the high and low dose mice with the high dose having a more significant increase. The graphs are shown in Figures 5.1.a and b. The mice used for this study were normal immune Balb-C mice and were therefore able to recover from infection without treatment. One high dose mouse however succumbed to the infection during the study.

Figure 5.1.a. Plot of raw CO/CO_2 by mouse status over time for Endotoxin LPS #1.

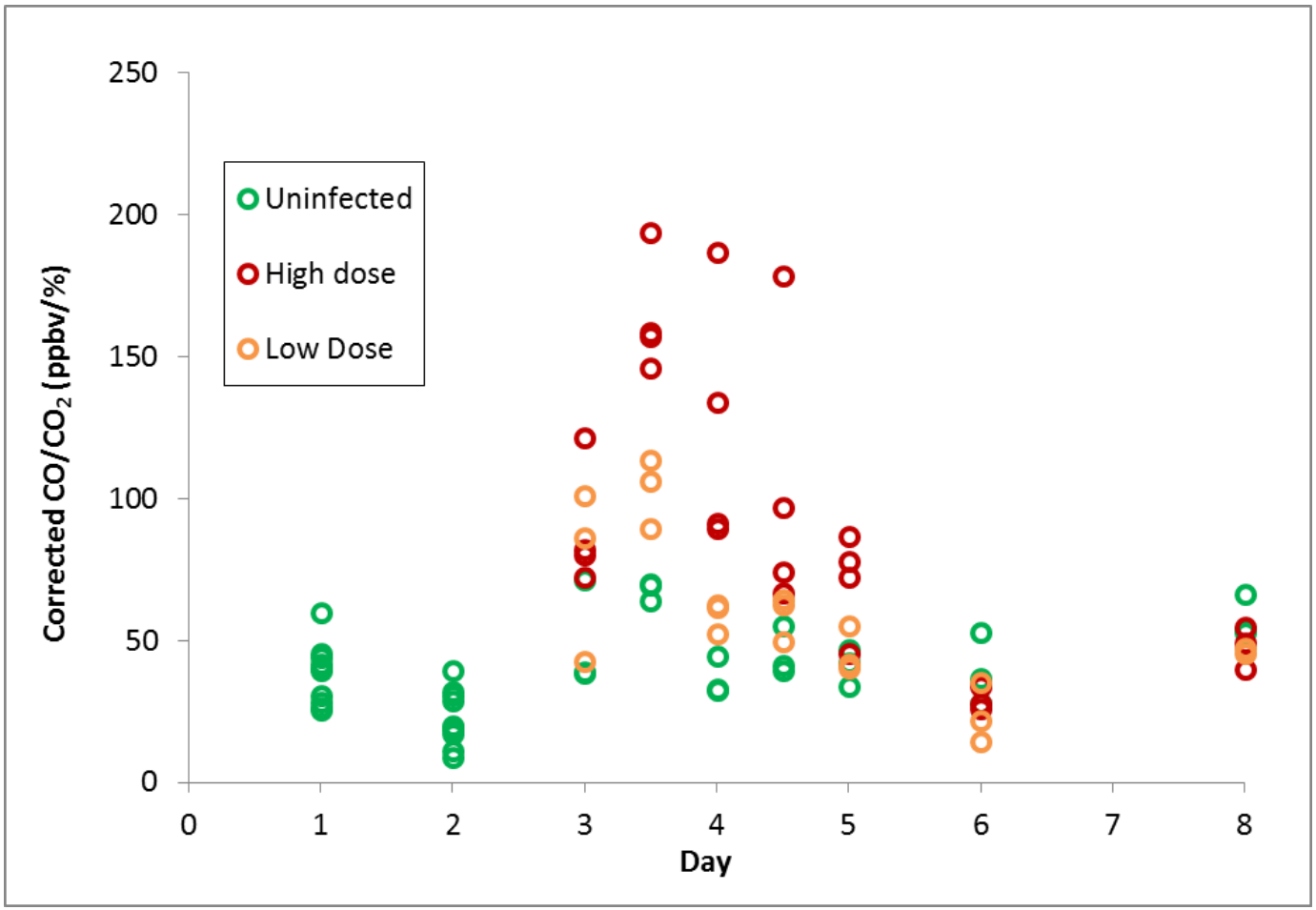

Figure 5.1.b. Plot of corrected CO/CO_2 by mouse status over time for Endotoxin LPS #1.

The average raw CO/CO_2 for the uninfected mice was 290 ppbv/% (192-384). For the low dose mice the average was 353 ppbv/% (212-546) and for the high dose mice it was 520 ppbv/% (218-1191). In the high dose mice, the mouse on day 5 that had a raw CO/CO_2 of 1191 ppbv/% was the mouse that expired later that evening. A t-test was performed on the uninfected vs. all infected samples over the study period and was significant with a p-value of $<10^{-4}$. In addition, t-tests were performed on the uninfected vs. low dose samples and uninfected vs. high dose samples with both being significant. The p-values for these t-tests were 0.01 and $<10^{-4}$ respectively. Finally, a t-test was performed on the low dose vs. the high dose samples and was also found to be significant with a p-value of $1.3*10^{-3}$.

The average corrected CO/CO_2 for the uninfected mice was 40 ppbv/% (9-71). The average for the low dose mice was 60 ppbv/% (14-113) and for the high dose was 94 ppbv/% (26-194). The p-value for the t-test on the uninfected vs. all infected samples was $<10^{-4}$. For the uninfected vs. low dose samples, the p-value was $4.8*10^{-3}$ and for the high dose samples, the p-value was $<10^{-4}$. The p-value for the t-test on the low dose vs. high dose samples was $5.1*10^{-3}$. This showed that there was a significant difference between all samples whether uninfected or infected with low or high dose after correction for blank concentrations.

As a means of controlling for individual mouse response, the raw CO/CO_2 concentrations for the high dose mice was plotted over time by mouse number with one of the uninfected mice shown as a reference. The results of this plot are shown in Figure 5.2. The asterisk indicates the mouse that expired prior to sampling on day 6.

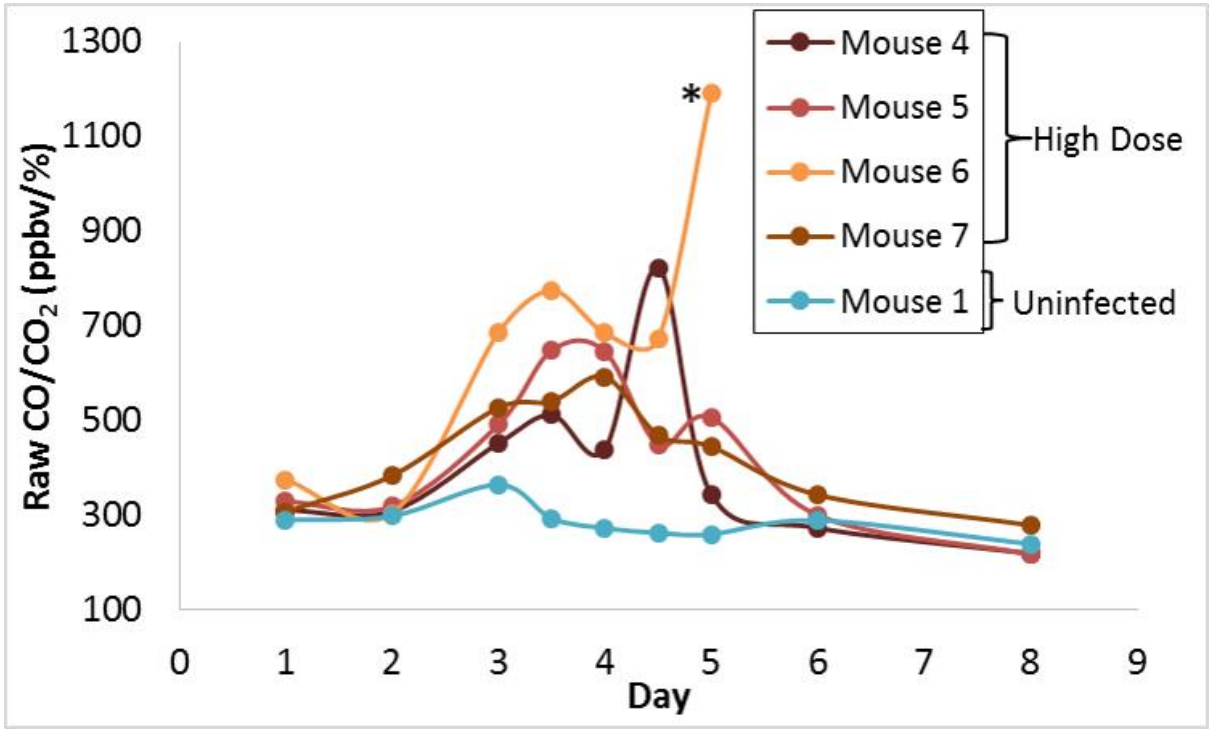

Figure 5.2. Plot of individual mouse raw CO/CO_2 concentration over time for high dose mice and one uninfected mice.

For the Endotoxin LPS #2 study, the CO and CO_2 concentrations were also determined. In this study, the mice were also separated into three groups this time as an uninfected group, a group that received an injection but were inherently resistant to the infection, and a group that received an injection and were sensitive to the infection. These groups were labeled as uninfected, resistant, and sensitive respectively. Again, plots were made of the raw CO/CO_2 concentrations over time as well as the corrected CO/CO_2 over time with colorized data points indicating status. The resulting graphs are shown in Figures 5.3.a and b.

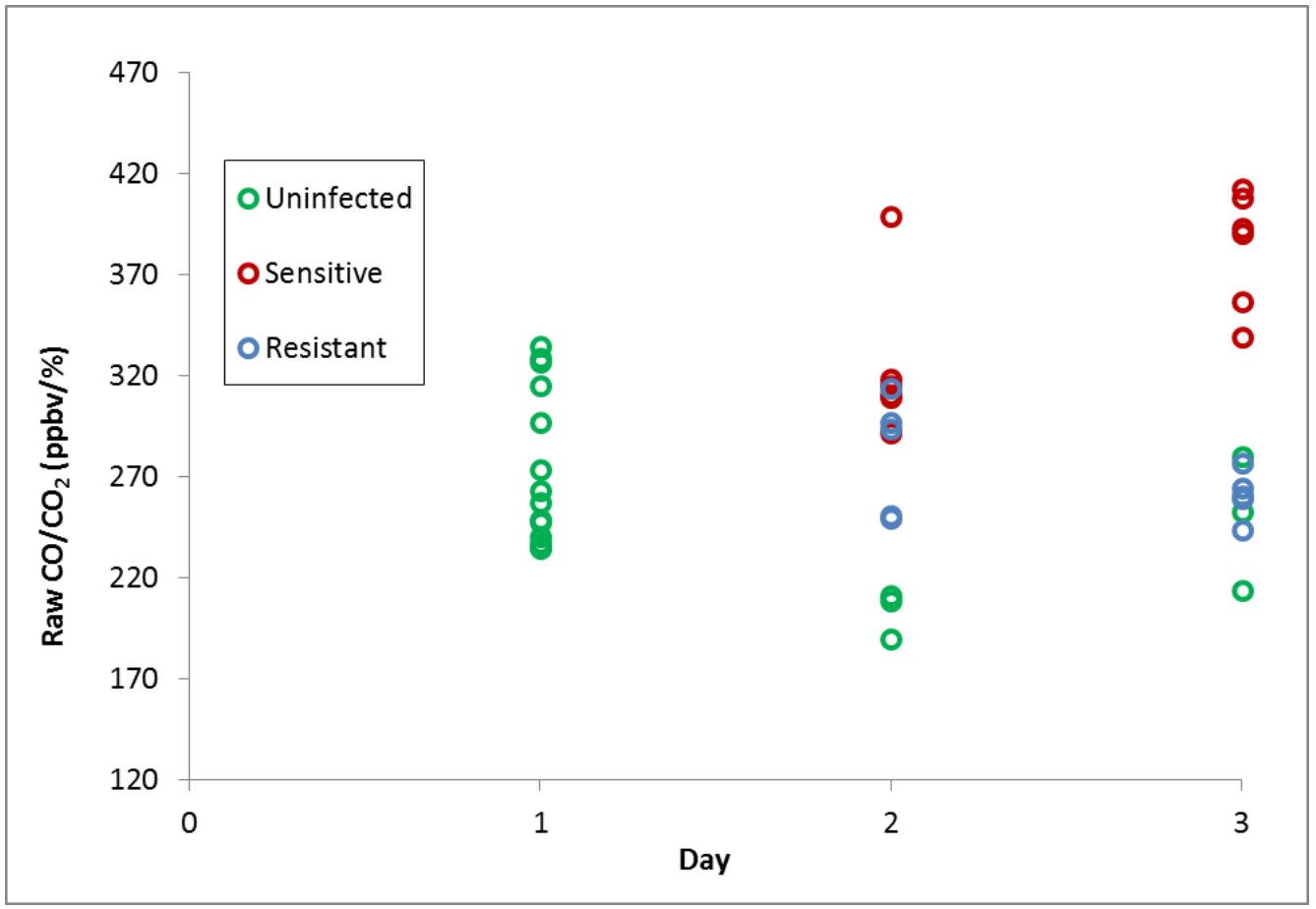

Figure 5.3.a. Plot of raw CO/CO_2 over time for Endotoxin LPS #2 study with color indicating status.

Figure 5.3.b. Plot of corrected CO/CO_2 over time for Endotoxin LPS #2 study with color indicating status.

The average raw CO/CO_2 concentration for the uninfected mice was 260 ppbv/% (190-334). For the resistant mice the average was 271 ppbv/% (244-314), while the average for the sensitive mice was 354 ppbv/% (292-412). As with the previous studies, t-tests were performed to determine the significance of the observed differences. For the uninfected vs. resistant data the p-value was 0.36 which was not significant, as expected. However, the uninfected vs. sensitive values were found to have a significant difference with a p-value of $<10^{-4}$. In addition, the difference between the resistant and sensitive mice was also found to be significant with a p-value of $<10^{-4}$.

For the corrected CO/CO_2 concentrations the average for the uninfected samples was 58 ppbv/% (31-83). The resistant samples had an average of 51 ppbv/% (32-69), and the sensitive samples average was 100 ppbv/% (53-137). The difference between the uninfected and resistant samples was still not significant with a p-value of 0.19. For the uninfected vs. sensitive values, the p-value was $2.8*10^{-4}$. The resistant vs. sensitive samples had a p-value of $<10^{-4}$. To demonstrate the change in the concentration of CO/CO_2 over time for individual mice, the concentrations over time were plotted for the sensitive mice with an uninfected mouse shown for reference. This graph is shown in Figure 5.4.

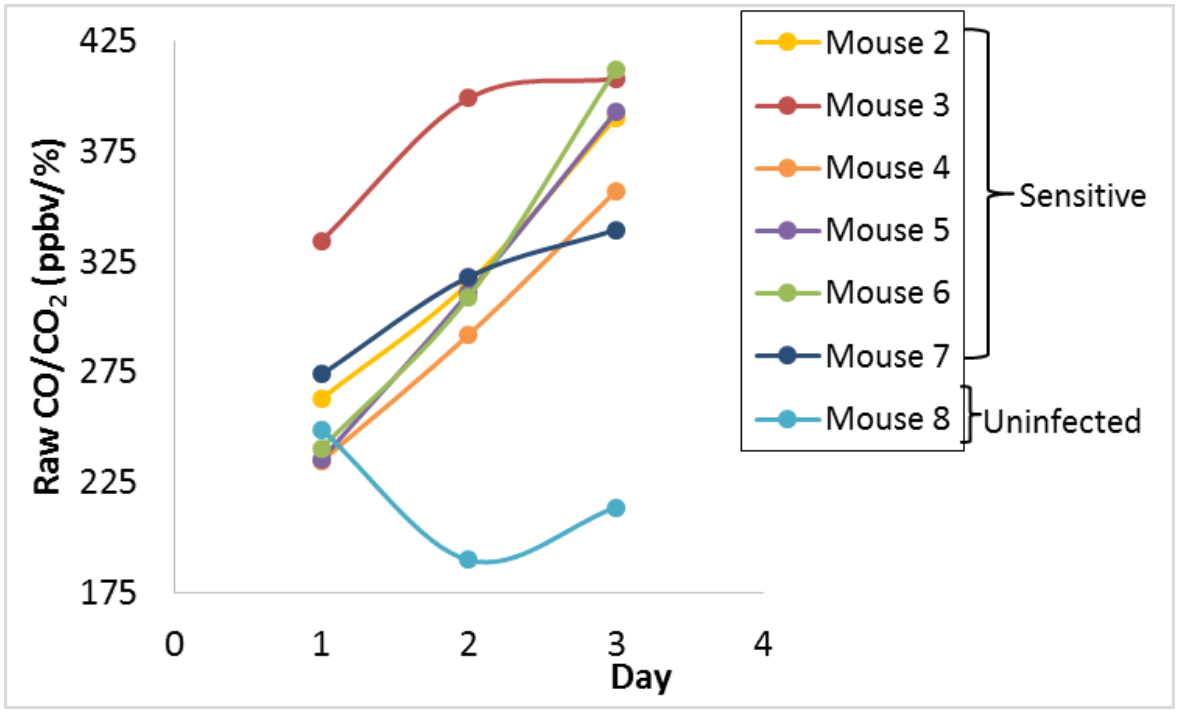

Figure 5.4. Plot of CO/CO_2 over time by mouse for sensitive samples and one uninfected.

The amount of heme-oxygenase-1 transcripts were measured in the serum of the mice at the end of this study as well. This enzyme increases in the blood as a response to hemolysis, which encourages the release of free radicals eventually leading to apoptosis. In addition, the number of actin transcripts were also measured, both by reverse transcriptase polymerase chain reaction (PCR). Because the actin transcripts are highly and consistently expressed, these values were used to normalize the amount of heme oxygenase measured. To do so, the number of heme oxygenase-1 transcripts was divided by the number of actin transcripts and the result was multiplied by 10,000. The CO/CO_2 was plotted vs. the result of the normalized heme oxygenase measurement and is shown in Figure 5.5.

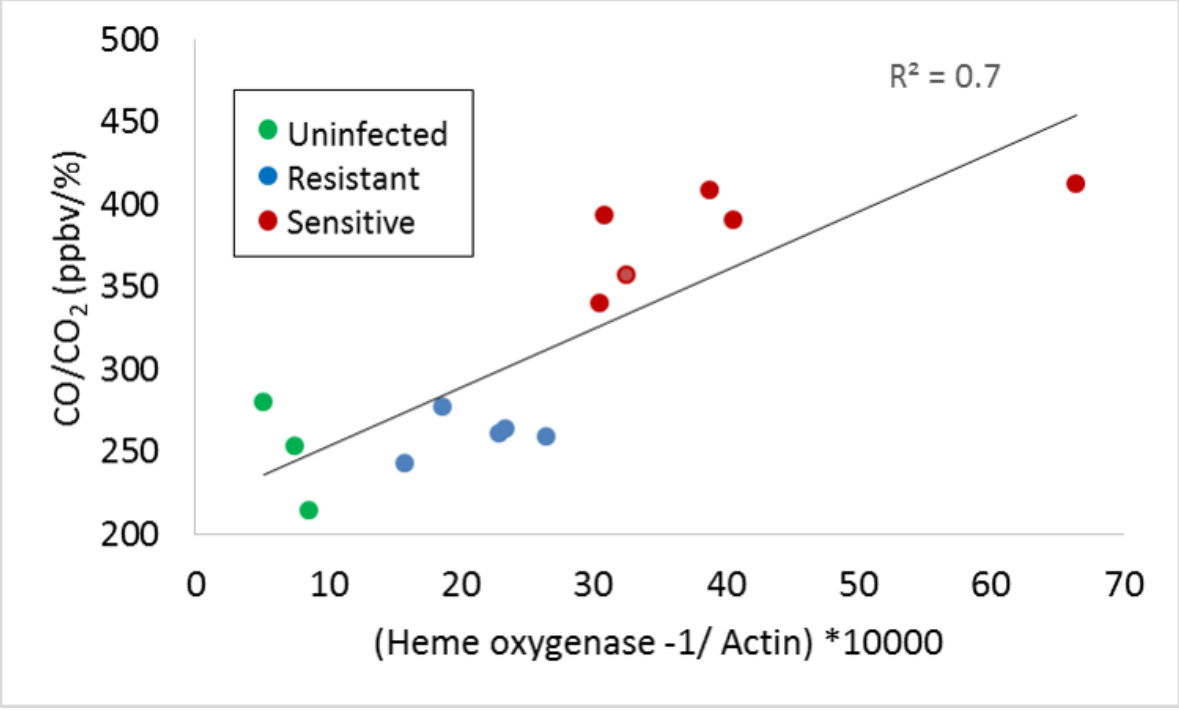

Figure 5.5. Plot of CO/CO_2 vs. normalized heme oxygenase-1 levels in blood of mice on the last day of the study.

For the third endotoxin study, Endotoxin OSP A, there were only two groups of mice. One group was uninfected for the duration of the study while the second group was infected with the outer surface protein A from *Borrelia burgdorferi*. For this study, the raw CO/CO_2 did not differ significantly over the study period. The average CO/CO_2 concentration for the uninfected samples was 210 ppbv/% (175-292). For the infected samples the average was 230 ppbv/% (194-265). To ensure there was no significant difference a t-test was performed on the uninfected vs. infected samples and was found to be not significant with a p-value of 0.07. Because there was no significant difference in the raw CO/CO_2 the corrected values will not be discussed.

The fourth endotoxin study, Endotoxin groups, was performed with groups of mice in the tower during sample collection. There were six groups of mice, three of which were uninfected throughout the study while the remaining three received injections of LPS endotoxin on the second day of the study. The raw CO/CO_2 was plotted over time for the uninfected and infected groups. Additionally, the corrected CO/CO_2 was plotted over time and the resulting graphs are shown in Figures 5.6.a and b.

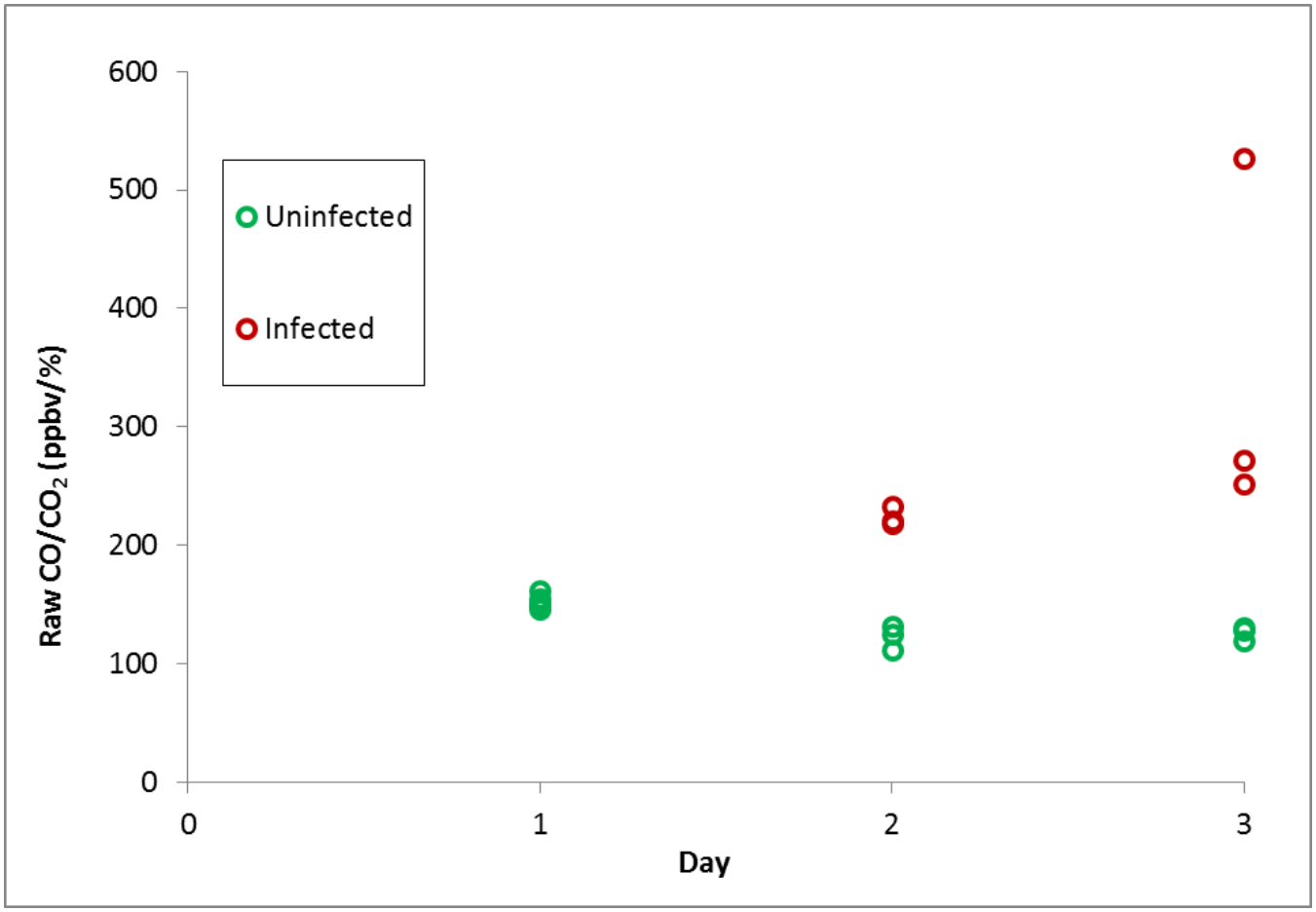

Figure 5.6.a. Plot of raw CO/CO_2 over time for Endotoxin groups study.

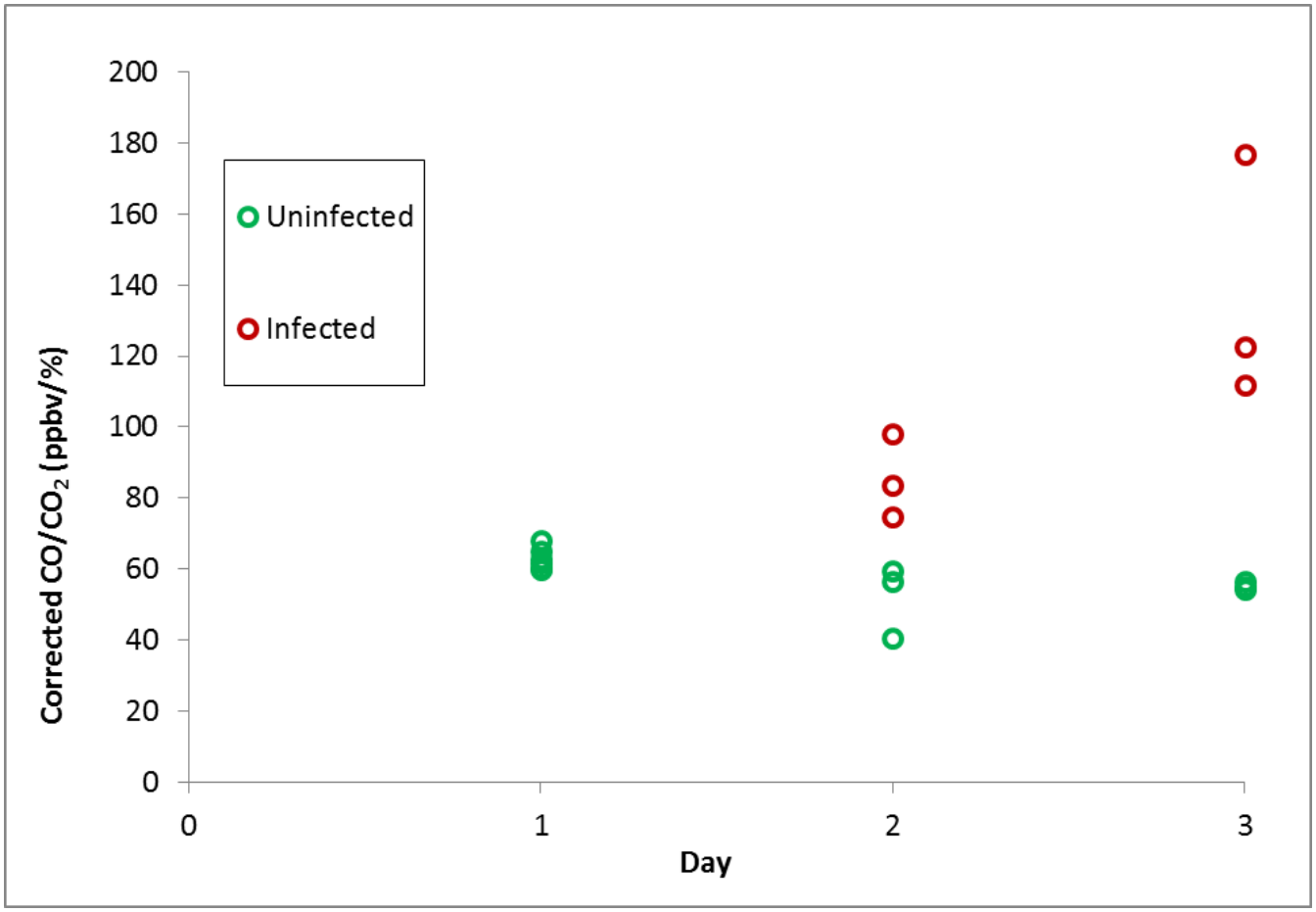

Figure 5.6.b. Plot of corrected CO/CO_2 over time for Endotoxin groups study.

For this study, the average uninfected raw CO/CO_2 concentration was 138 ppbv/% (112-162). For the infected groups the average raw CO/CO_2 was 287 ppbv/% (219-527). A t-test was performed on the uninfected vs. infected samples for both days of infection and the resulting p-value was 0.03. In addition, t-test were performed on the uninfected samples and infected samples collected on day two of the study and the p-value was found to be $2.3*10^{-4}$. A similar t-test performed on the results of day three however was surprisingly found to be not significant with a p-value of 0.13. This can likely be attributed to two factors, the first being the small number of samples (n=6). Also, on the third day one of the infected groups had a significantly low CO_2 value compared to the rest of the infected groups, 0.22% vs. an average infected CO_2 concentration of 0.51%. This caused the CO/CO_2 value for this group to be much higher than the other two groups on day three at 527 ppbv/% (compared to 252 and 271). In fact, if this high ratio was instead replaced with the average of the other two groups on day three the p-value became significant at $1.5*10^{-4}$.

The average corrected CO/CO_2 for the uninfected groups was 58 ppbv/% (41-68). The infected average corrected CO/CO_2 was 111 ppbv/% (75-177). The t-test performed on all uninfected vs. infected samples had a p-value of 0.02. A t-test performed on the day two samples also had a p-value of 0.02. Again the t-test for the third day was found to be not significant, likely due to the large variation in CO_2 concentrations between the groups, with a p-value of 0.06. If however the CO/CO_2 value (177 ppbv/%) for the group with the low CO_2 concentration was replaced with the average of the other two groups (112 and 123 ppbv/%), the p-value became $1.6*10^{-3}$. As in the previous studies, a plot was made showing the effect on the individual groups as well and is shown in Figure 5.7.

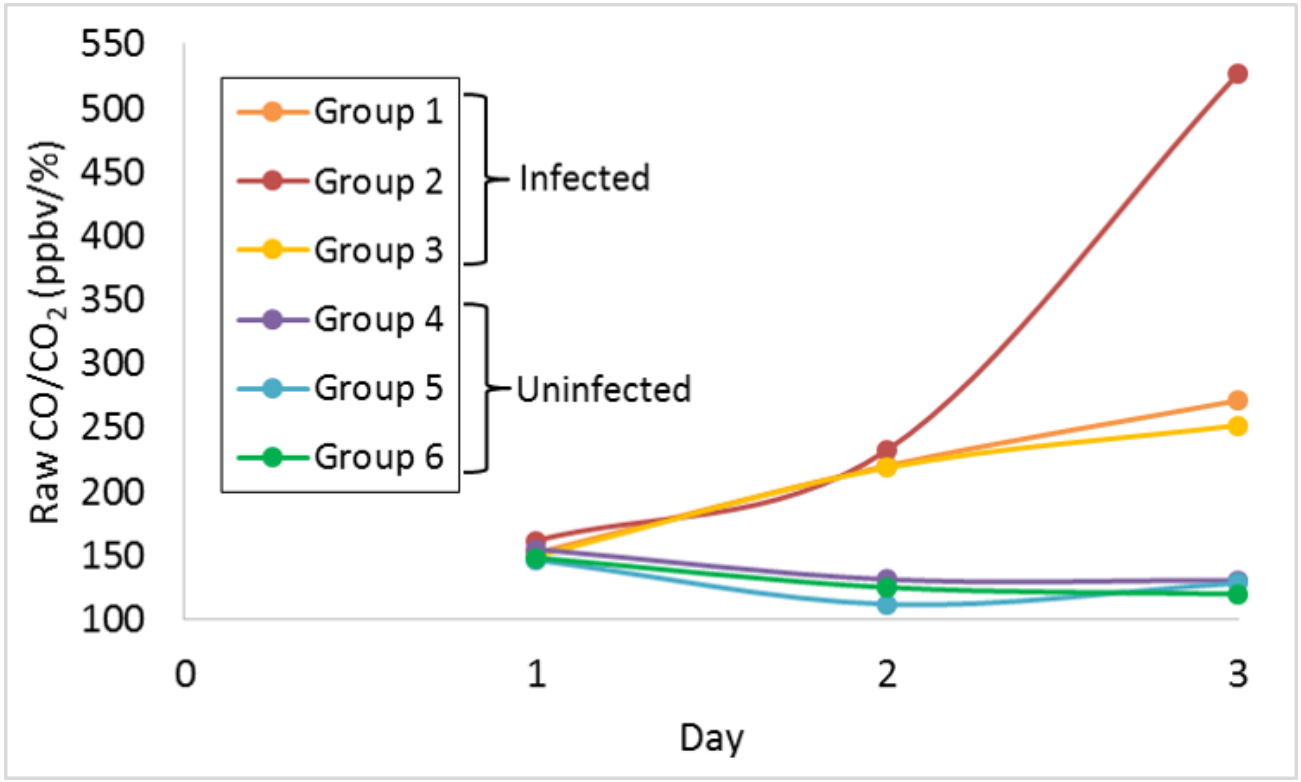

Figure 5.7. Plot of CO/CO_2 over time separated by groups of mice for Endotoxin group study.

The final endotoxin study was performed on individual rats. There were four rats that were uninfected for the first six days of the study after which all four received injections of LPS endotoxin. Since the rats were all infected the plot of the raw CO concentration over time was made by individual rat with the date of infection indicated on the graph. A similar plot was made for the corrected CO over time as well as the corrected CO/CO_2 over time. The resulting graphs are shown in Figures 5.8.a, b, and c.

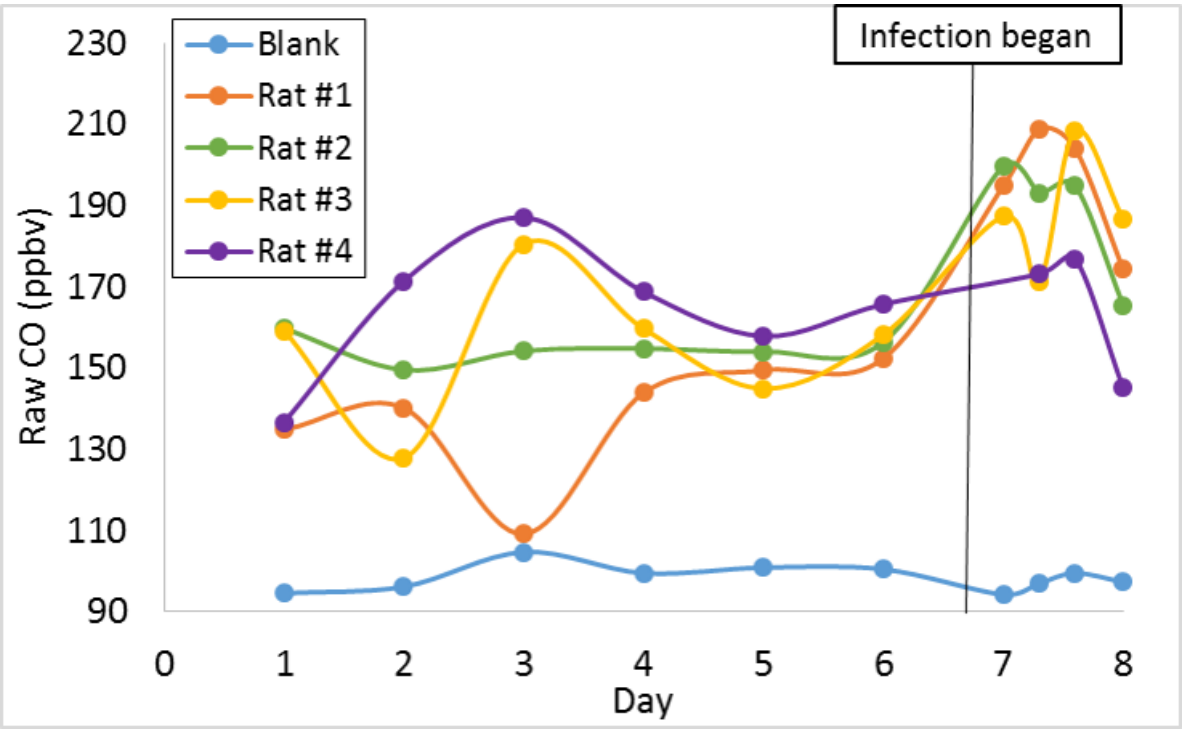

Figure 5.8.a. Plot of raw CO over time for individual rats from Endotoxin rats study.

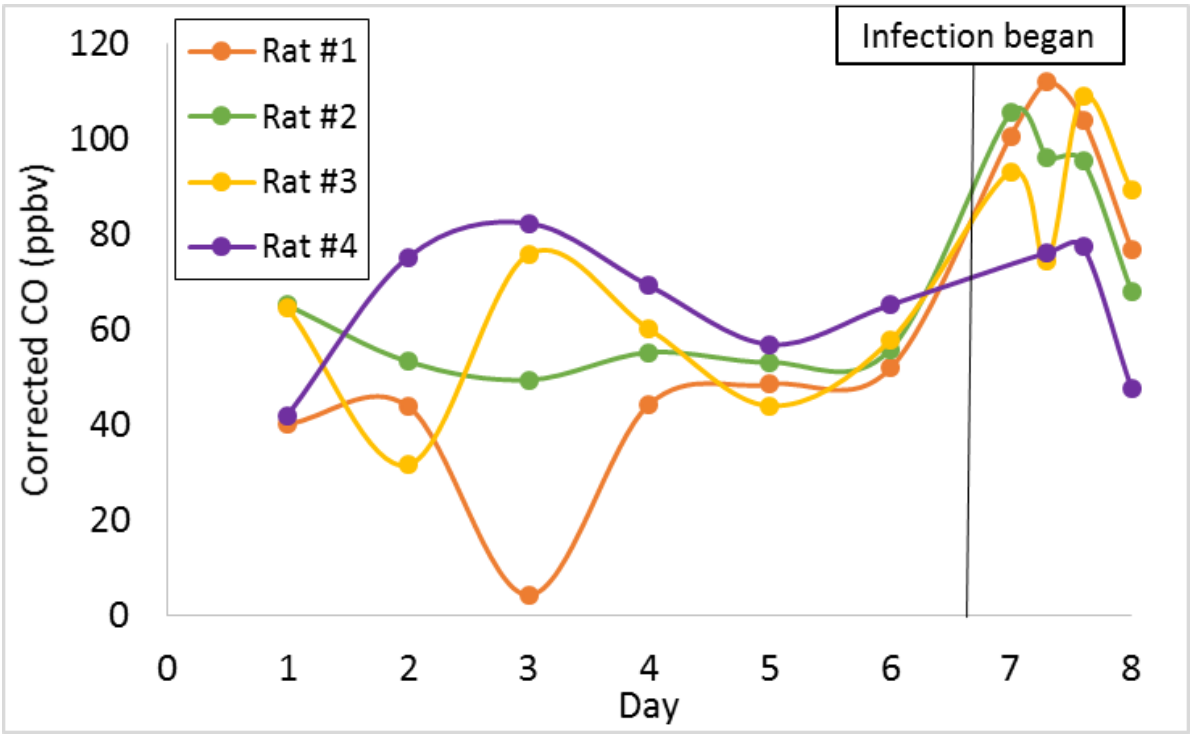

Figure 5.8.b. Plot of corrected CO over time for individual rats from Endotoxin rats study.

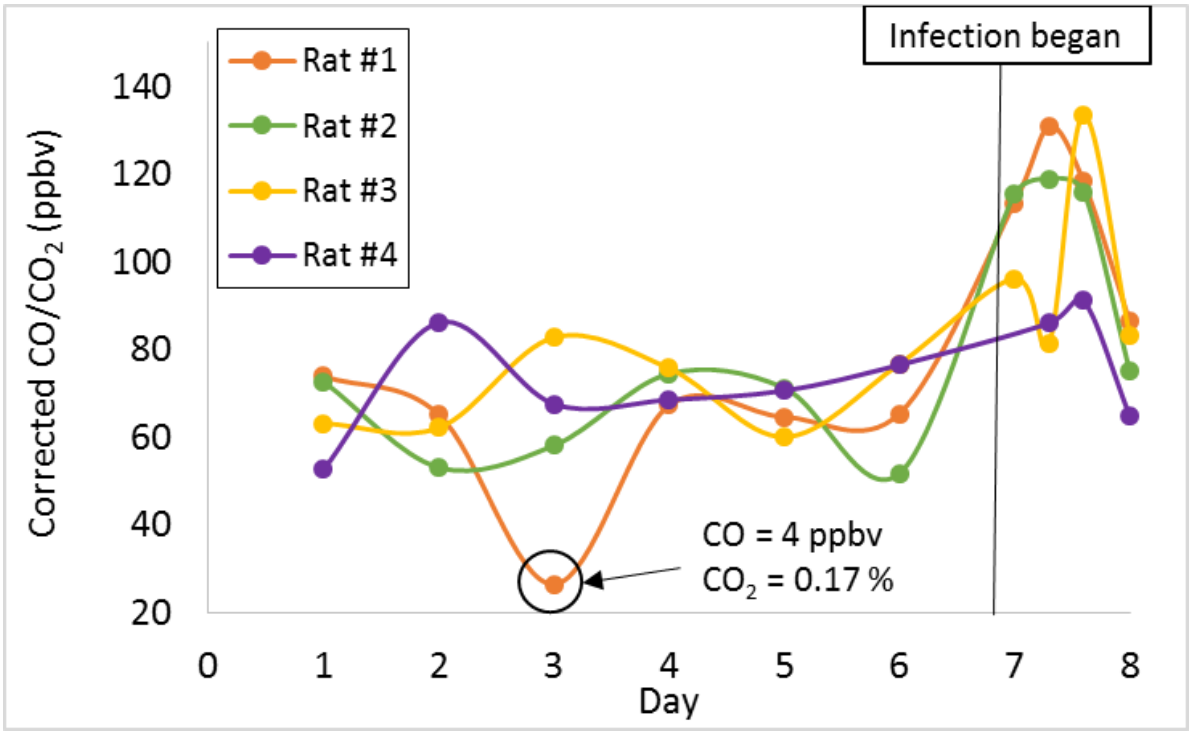

Figure 5.8.c. Plot of corrected CO/CO_2 over time for individual rats from Endotoxin rats study.

For the raw CO concentrations, the average of the days before infection began was 153 ppbv (109-187). For the samples collected after infection began, the average raw CO concentration was 186 ppbv (145-209). The average corrected CO concentration for the days prior to infection was 54 ppbv (4-82) and for the samples after infection the average was 88 ppbv (48-112). For the corrected CO/CO_2 the average on the days before infection was 66 ppbv/% (27-86), and for the infection days it was 101 ppbv/% (65-134).

On day three of sampling, rat #1 had low CO and CO_2 concentrations, likely due to turning during sampling. This point is indicated with a circle on the corrected CO/CO_2 graph and the corrected CO and CO_2 concentrations are labeled for that sample. This demonstrated the ability to determine a "bad" vs. "good" breath sample based on the concentration of CO_2. Based on the results in the graphs, it appeared that rat #4 did not get very sick from the infection. Also, by day eight of sampling all the rats seemed to have recovered from the infection, based on the CO and CO/CO_2 values. For these reasons, the t-tests were performed in two ways, first with all four mice and all samples after infection being considered as infected, then again with mouse four and day eight considered as uninfected. The results of these t-tests are shown in Table 5.1.

Table 5.1. Results of t-tests for rat tower data for raw CO, corrected CO and CO/CO_2.

	T-test all rats infected days 7 and 8	**T-test rat 4 uninfected rats 1-3 infected only day 7**
Raw CO	$4.3*10^{-6}$	$2.9*10^{-7}$
Corrected CO	$1.5*10^{-6}$	$1.2*10^{-7}$
CO/CO_2	$1.4*10^{-5}$	$5.3*10^{-6}$

In addition to the tower study, the rats were sampled in a glass bulb to compare to the results obtained with the tower. The CO and CO_2 concentrations were determined and

analyzed. When comparing the tower and bulb values, the blank concentrations were very similar. For the tower the average CO concentration was 98 ppbv while the average for the bulb was 94 ppbv. The average blank CO_2 concentration for the tower was 0.04% and for the bulb was 0.03%. The concentrations for the rats however varied between the tower and bulb. This was likely a result of the re-breathing of the air in the bulb by the rats over time because of the lack of constant flow of background air, as was present in the tower. The average raw CO for the tower for all rats throughout the study was 166 ppbv, while the average for the bulb was 227 ppbv. For the raw CO_2 the average in the tower was 0.87% and that of the bulb was 1.09%. After correcting for the blank, the average CO concentrations were 67 ppbv and 137 ppbv for the tower and bulb respectively. The average corrected CO_2 concentrations for the tower was 0.83% and for the bulb it was 1.06%.

Averages for the bulb data were also taken for the rats before infection and compared to averages after infection began. For the raw CO, the average before infection was 230 ppbv (199-256) and the average after was 219 ppbv (203-246). The average for the corrected CO before infection was 136 ppbv (104-163) and after infection began the average was 125 ppbv (107-153). The average corrected CO/CO_2 before infection was 128 ppbv/% (105-154), and after it was 117 ppbv/% (96-130). Unlike the tower results, there was no significant difference between the samples collected before and after infection. This was evident not only in the averages but also by the t-test results for which the p-values for raw CO, corrected CO, and CO/CO_2 were 0.40, 0.93, and 0.41 respectively.

Discussion: CO and CO_2

For both of the studies using individual mice infected with LPS endotoxin as well as the studies using groups of mice and the study on individual rats in the breath tower, a significant increase in the CO/CO_2 concentrations was seen over time. For the OSP A endotoxin study however, there was not a significant difference observed over time which could be a result of either the short sampling time, the mice not having gotten infected, or a combination of both. In addition, the study using the glass bulb with the rats infected with LPS endotoxin did not show a significant change in the CO/CO_2 over time which could have been an effect of the rebreathing of the air in the bulb by the rats during the sampling period. While this rebreathing lead to a higher overall average of both the CO and CO_2 collected from the rats in the bulb, it also seemed to mask the increase in the CO and CO/CO_2 in the infected rats that was seen in the tower samples.

Results: NMHC analysis

There were two studies that were analyzed for NMHCs for the endotoxin projects, the study using groups of mice, Endotoxin groups, and the study using individual rats. The other studies were only analyzed for CO and CO_2 because of the small amount of breath sample collected which would not produce high enough concentrations of other VOCs for a difference to be seen between the background air and breath samples. For the two studies, all samples were analyzed for various VOCs using the Rowland Blake NMHC system as described in the methods section.

For the first of the two studies, Endotoxin groups, the results of the VOC analysis were analyzed for differences using a plotting program which allowed for the uninfected and infected samples to be plotted over time for each VOC that was measured. The graphs

were analyzed first for the raw data with the blank plotted in addition to the breath samples, and again for the corrected and CO_2 divided samples. Unlike the salmonella groups study, the CO_2 results for the infected mice were significantly lower than the uninfected samples. In fact, the average corrected CO_2 concentration for the uninfected mice was 1.01%, while that of the infected mice was only 0.47%. This led to difficulty in analysis because of the fact that at this low concentration of breath in the infected samples, the concentration of many VOCs was not high enough for a difference to be seen between the breath and blank samples. This can be seen in Figure 5.9 which shows overlaid chromatograms of the n-pentane peak for two infected samples, a blank sample, and an uninfected sample.

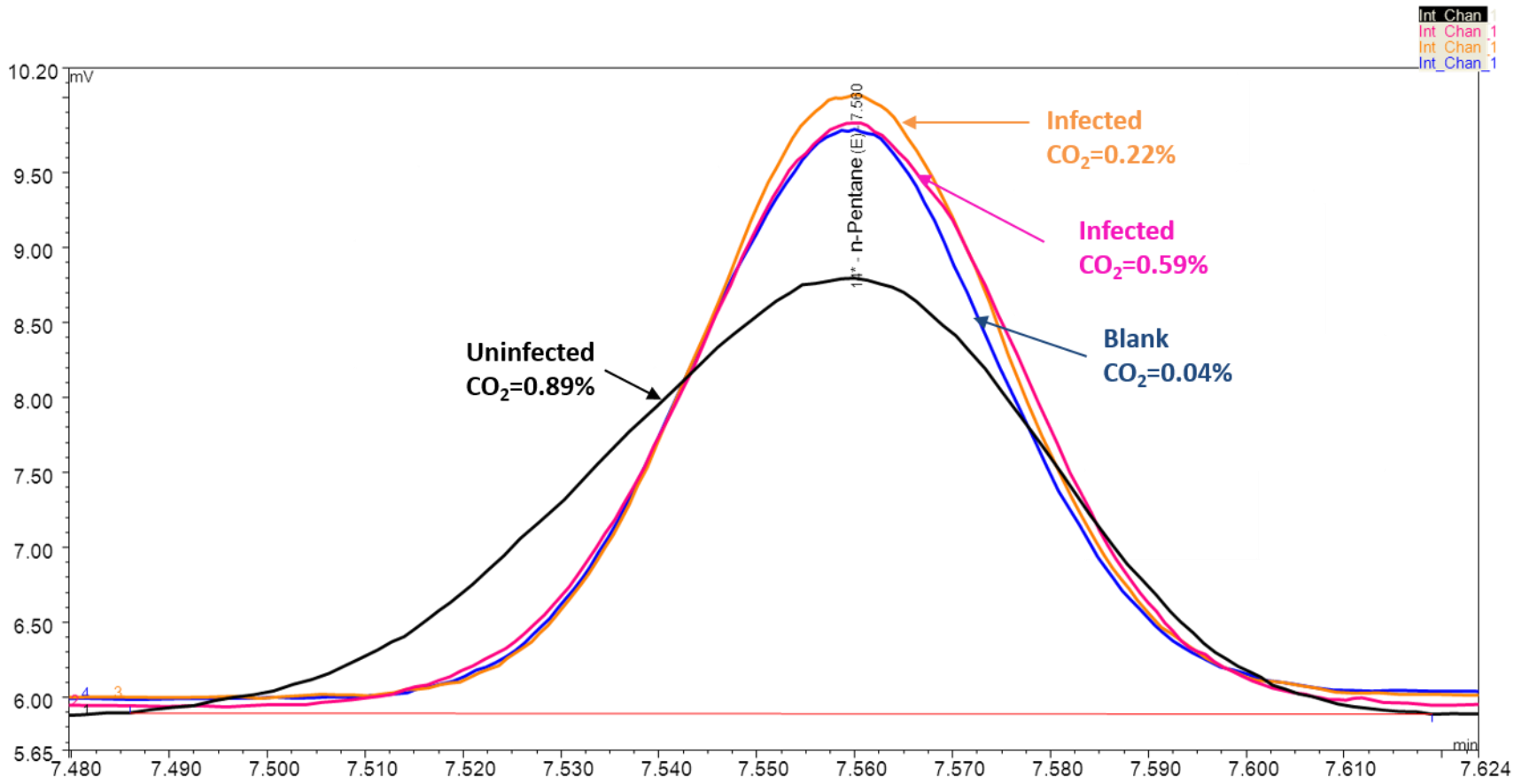

Figure 5.9. Overlay of chromatograms for n-pentane peak showing two infected (orange and pink), one blank (blue), and one uninfected sample (black).

As can be seen in the Figure above, the amount of CO_2 in the breath sample will not only affect the concentration of the determined gases, but can also affect the shape of the peak for some gases. This is most evident in the "spread out" peak that was observed with the uninfected sample when compared to the blank and infected samples. In addition, the two infected samples with concentrations of $CO_2 < 0.6\%$ are similar in both size and shape to that of the blank which contained only 0.04% CO_2. This would suggest that the amount of breath collected in the infected samples was not sufficient for a difference to be seen in the concentration of this VOC.

While the results for many of the VOCs measured for this project were similar to those observed with the n-pentane peak one gas, i-pentane, was identified for possible differences in the infected and blank samples. Over the course of the study, the average raw i-pentane concentration for the uninfected samples was 59 pptv. For both days of infection, the average concentration for the infected samples was 84 pptv. On the second day of the study (first infected day) the average for the infected samples was 74 pptv, and on day 3 it was 93 pptv. For the corrected and CO_2 divided concentrations, the uninfected average was -18 pptv while the infected averages were 20 pptv for both days, with a concentration of 5 pptv on the second day, and 35 pptv on the third day. T-tests were performed on the uninfected vs. infected samples for both the raw and corrected/CO_2 divided concentrations and had p-values of $6.5*10^{-3}$ and $8.4*10^{-3}$ respectively. The graphs resulting from the plotting program for the raw and corrected i-pentane are shown in Figures 5.10.a and b.

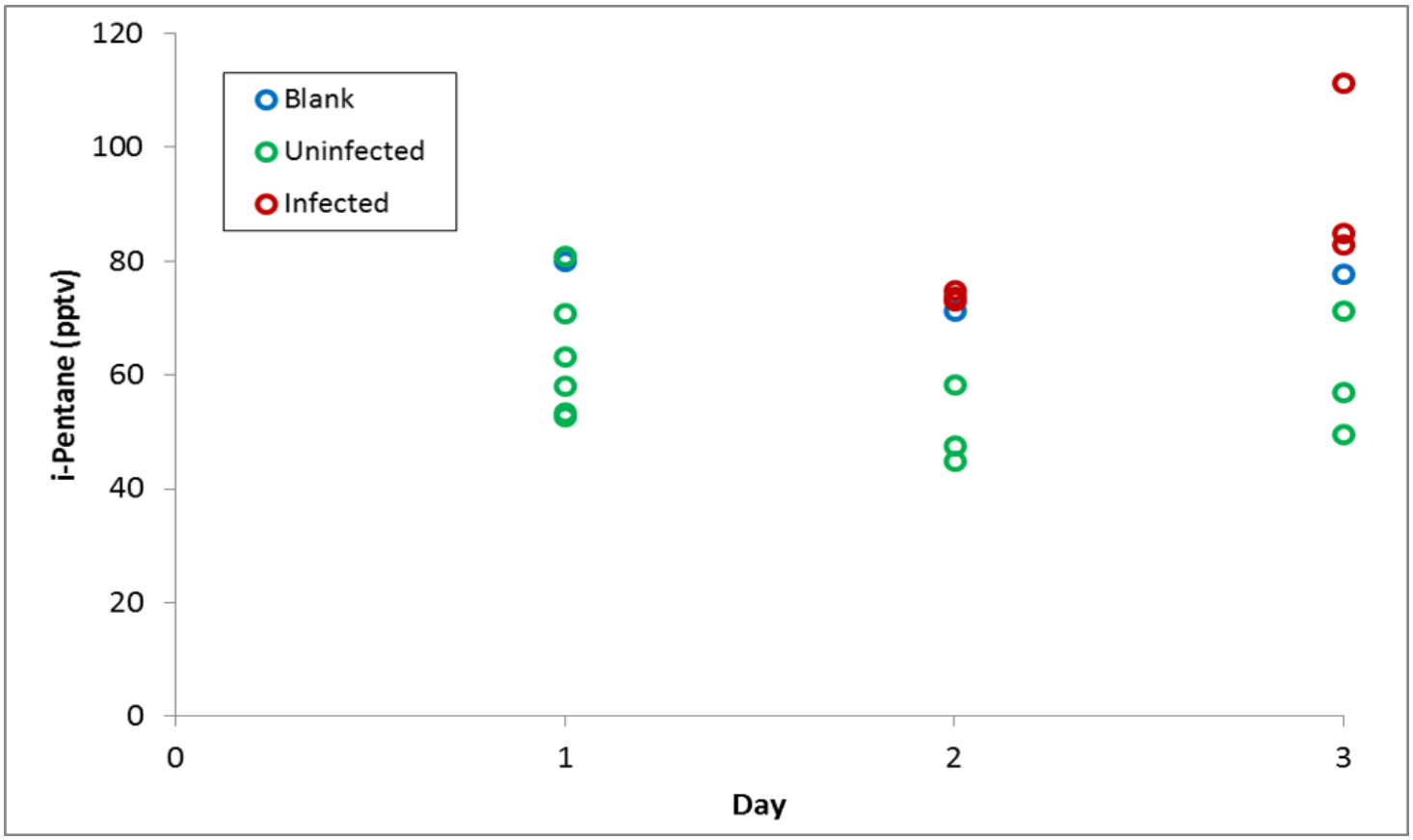

Figure 5.10.a. Plot of raw i-pentane concentration over time for infected and uninfected samples.

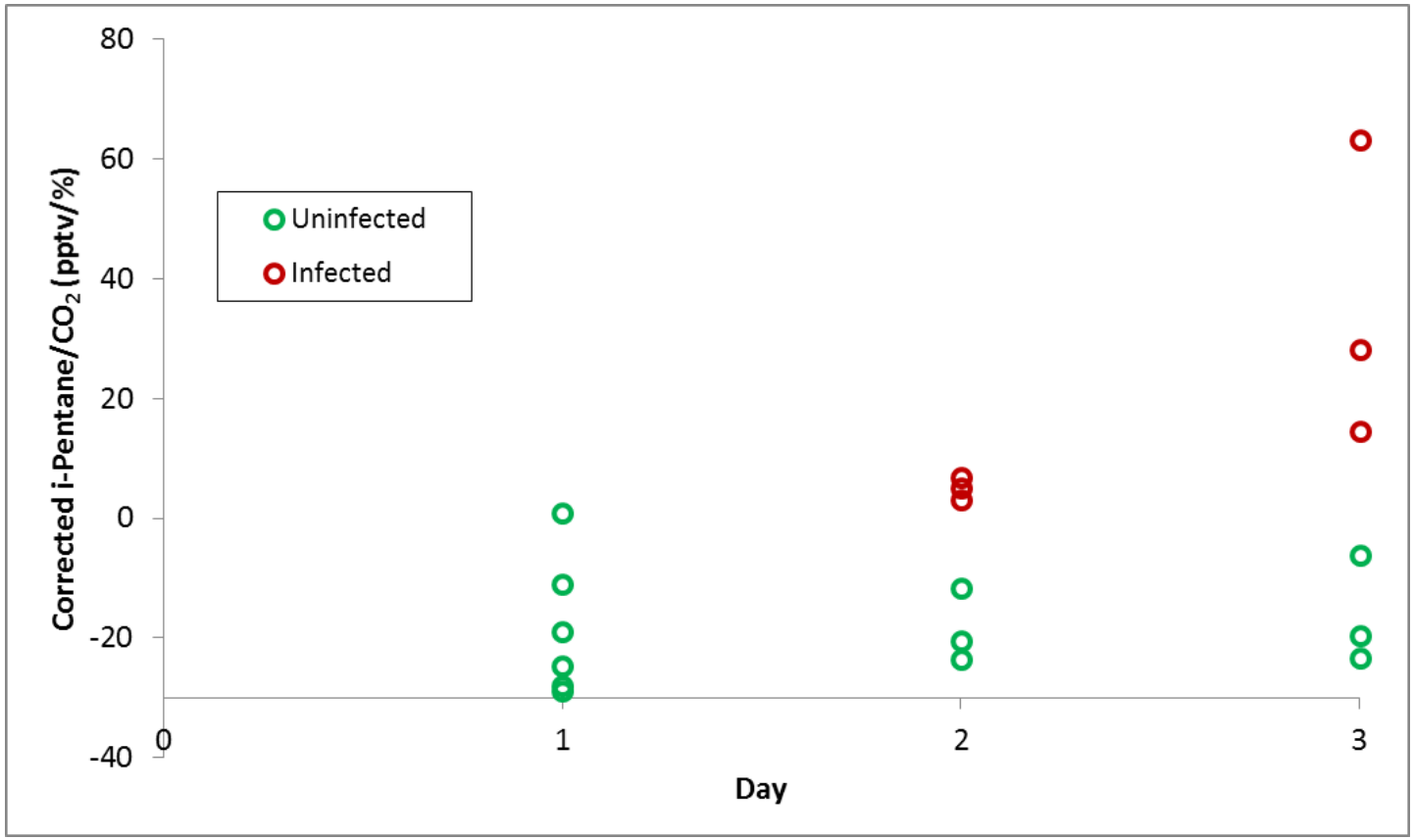

Figure 5.10.b. Plot of corrected i-pentane concentration over time for infected and uninfected samples.

The second of the two studies analyzed for NMHCs was the Endotoxin rats study. This study was performed on individual rats in both the breath tower as well as in a glass bulb. Both the tower and the bulb samples were analyzed for various VOCs using the NMHC system in the Rowland Blake lab. The results were analyzed to determine if differences could be seen between the uninfected and infected samples over the course of the experiment.

For the tower samples, there were two gases that were identified as showing a difference in the uninfected and infected samples. The first gas was acetone. The average acetone for the blank was 17 ppbv (10-19). For the samples collected before infection, the average acetone concentration was 39 ppbv (22-53). After infection began, the average acetone concentration was 53 ppbv (31-96). On the day of infection (without day 8), the average was 56 ppbv (39-96). The p-value for the t-test on the samples collected before and after infection began was $6.9*10^{-3}$. The average corrected and CO_2 divided acetone concentration for the samples before infection was 27 ppbv/% (15-41). After infection began, the average corrected acetone was 43 ppbv/% (17-97), with a day of infection average of 48 ppbv/% (23-97). The p-value for the t-test on the corrected acetone concentrations was 0.01. The raw and corrected acetone concentrations were plotted over time by individual rat to visualize the difference throughout the study. These graphs are shown in Figures 5.11.a and b.

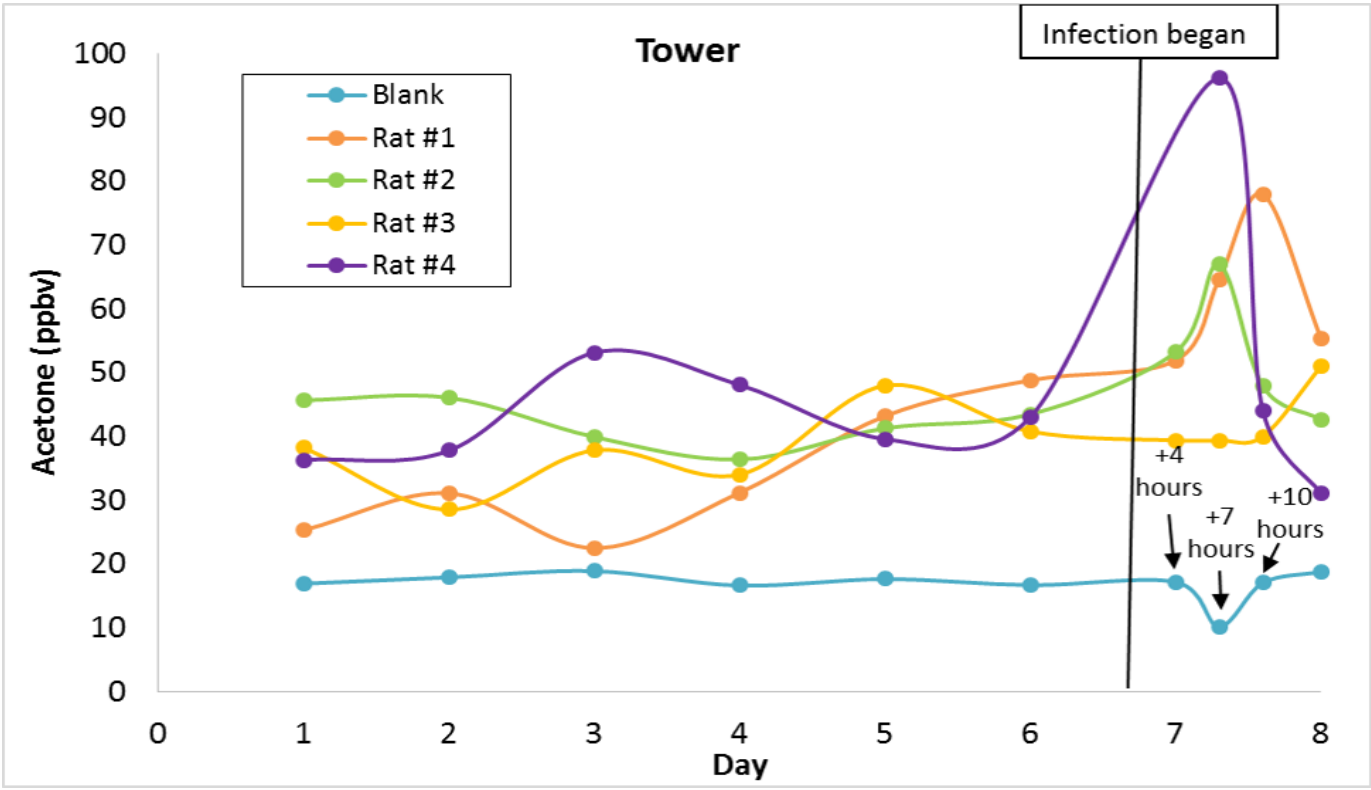

Figure 5.11.a. Plot of raw acetone concentrations overtime for blank and individual rats.

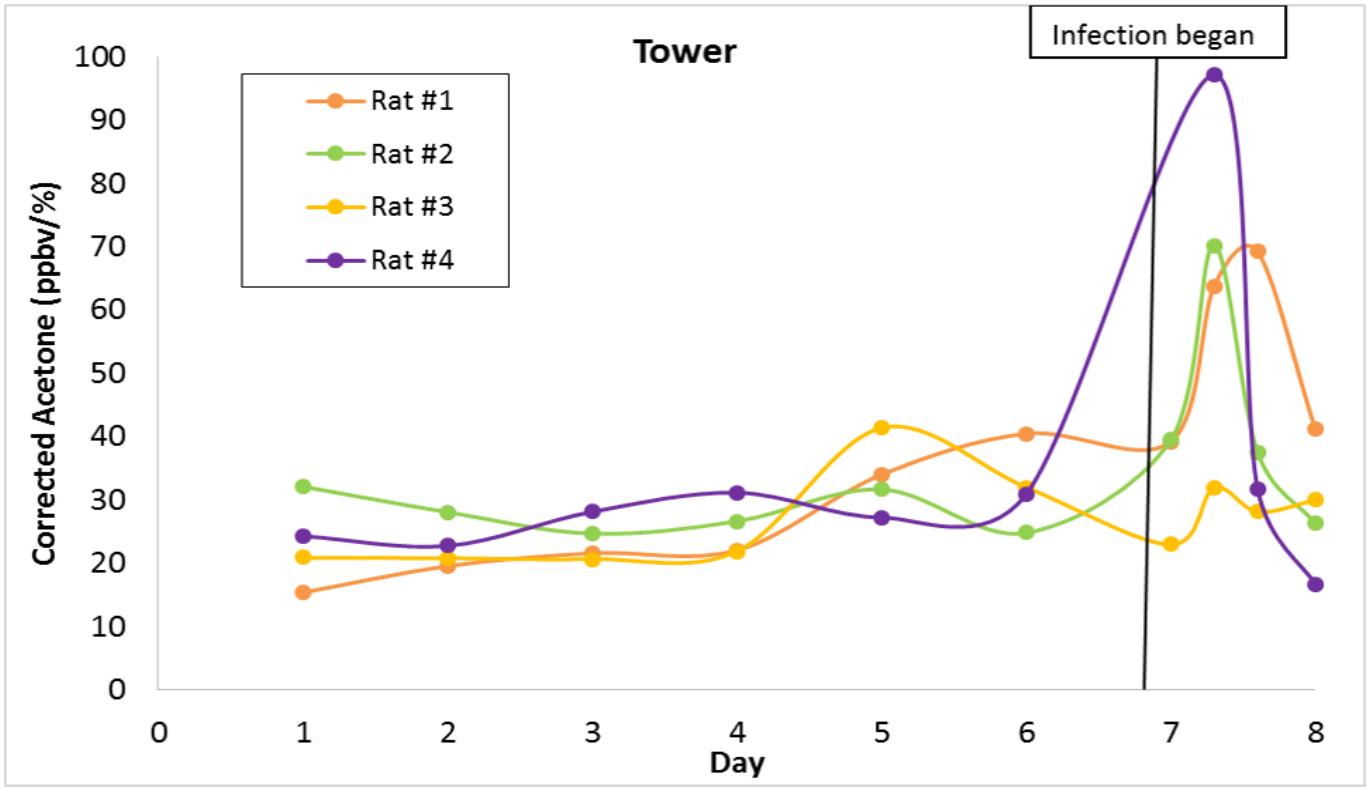

Figure 5.11.b. Plot of corrected acetone concentrations over time for individual rats.

The second gas identified as having a difference in the tower samples was an unknown. The average area determined from the chromatograms of the samples collected before infection was 114 (46-239). After infection began, the average was 2,900 (76-12,401). A t-test was performed on the samples before and after infection began and the p-value was 0.02. For the samples collected on the day infection began, the average was 3,800 (867-12,401). After subtraction of blank values and division of CO_2 concentrations, the average before infection became -23 (-535-309). After infection began the average corrected values were 3100 (44-12,745). On the day of infection, the average was 4,200 (888-12,745). The p-value for the t-test on the corrected values was 0.01. As with acetone, plots were made of the individual rats over time for both the raw and corrected values. The resulting graphs are shown in Figure 5.12.a and b.

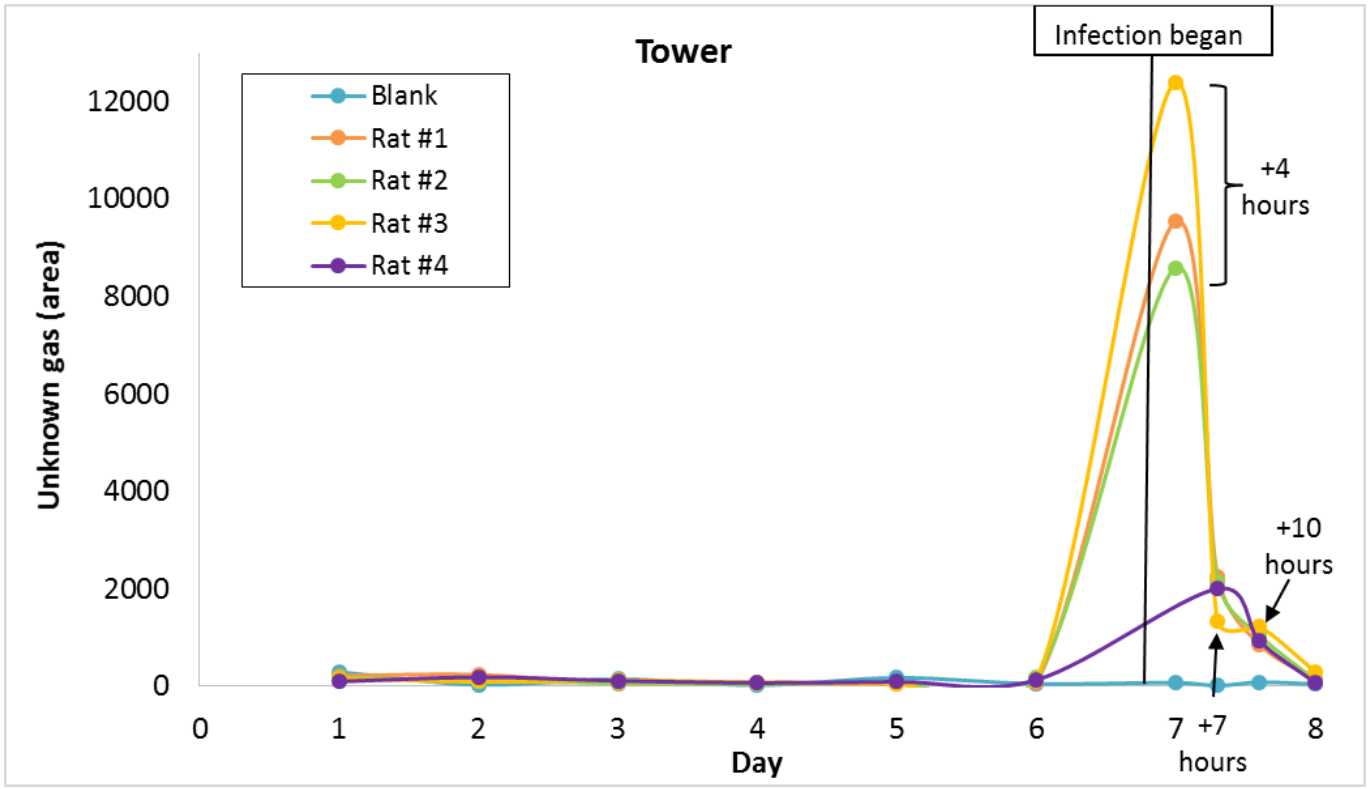

Figure 5.12.a. Plot of raw unknown gas over time for blank and individual rat samples.

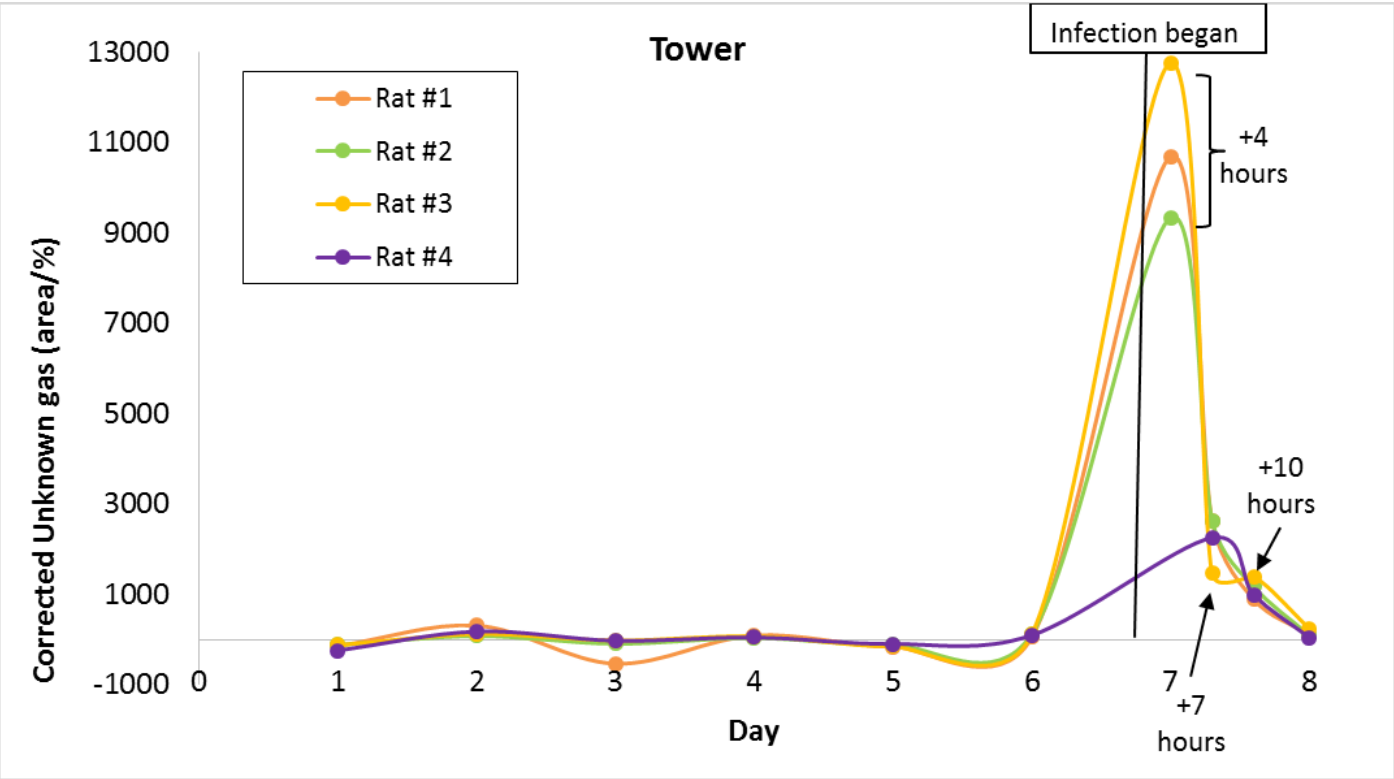

Figure 5.12.b. Plot of corrected unknown gas over time for individual rats.

The samples collected in the bulb were also analyzed for differences. For these samples, the acetone and unknown gas appeared different in the plots over time however the t-test did not show a significant difference. There was a smaller sample size in the case of the bulbs however which may be the reason for the t-test not showing a difference. For the acetone concentrations, the average for the samples collected before infection was 62 ppbv (38-100). After infection began the average acetone was 164 ppv (87-310). The p-value for the t-test was 0.14. For the corrected concentrations, the average before infection was 55 ppbv/% (40-82). The average after infection began was 151 ppbv/% (86-309). The p-value for the t-test was 0.17. The concentrations of acetone over time were plotted by individual rat for both the raw and corrected data. The resulting graphs are shown in Figures 5.13.a and b.

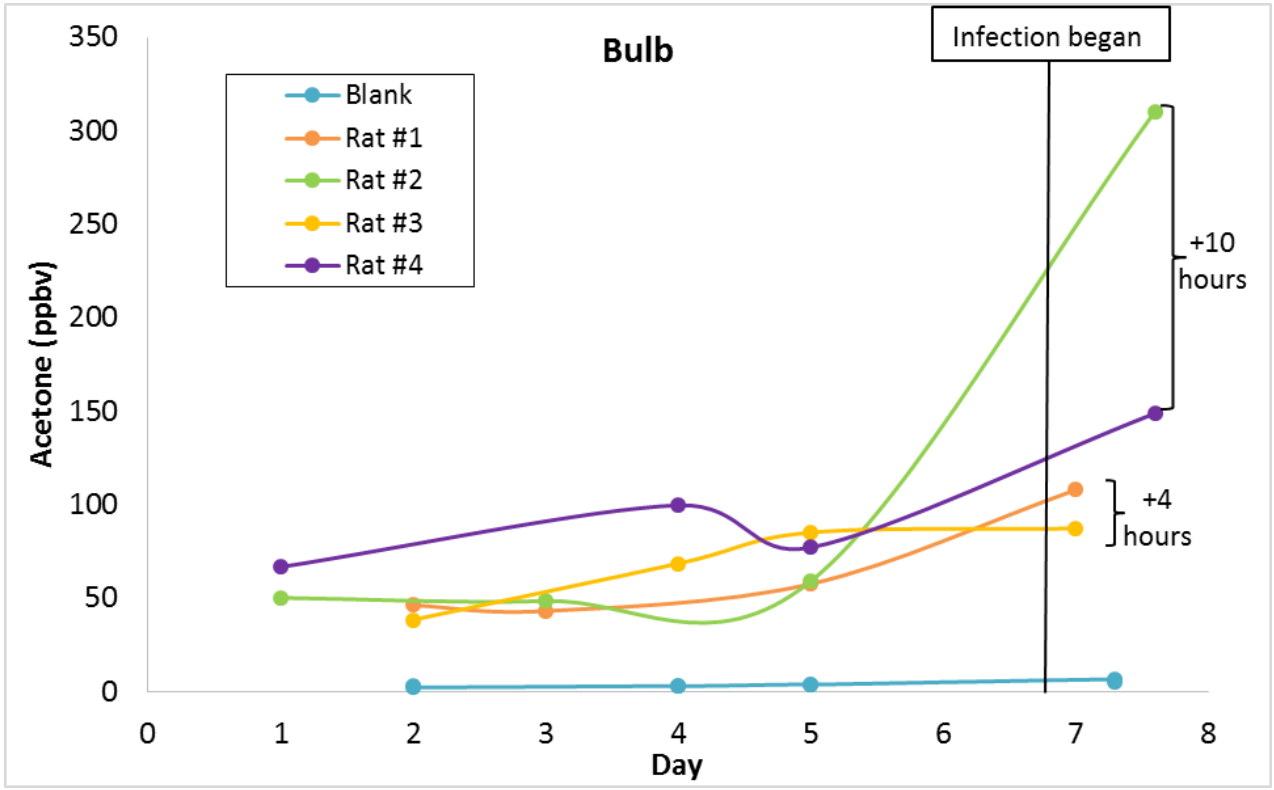

Figure 5.13.a. Plot of raw acetone concentrations over time for blank and individual rats in glass bulb.

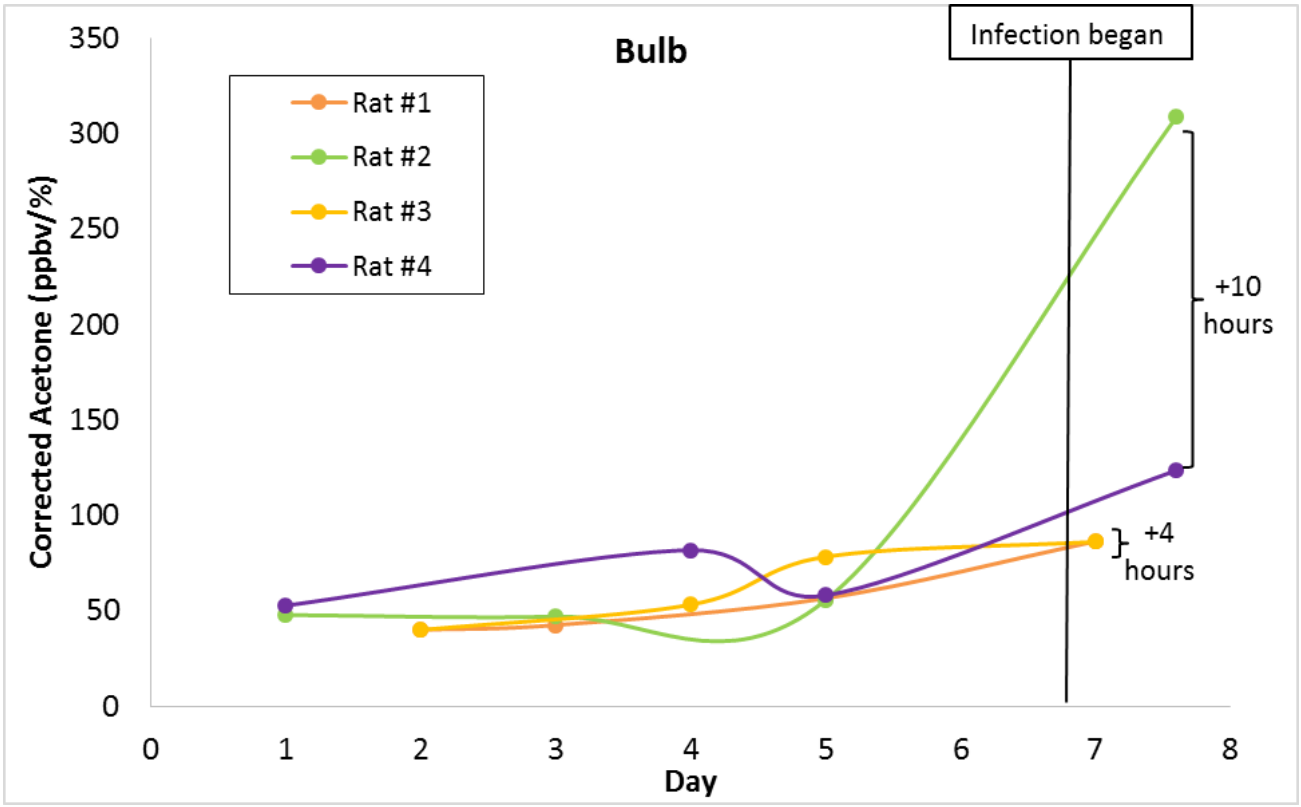

Figure 5.13.b. Plot of corrected acetone concentrations over time for individual rats in glass bulb.

The unknown gas had an average value in the bulb before the infection began of 44 (34-153). The average after infection began was 7,600 (1300-15,368). The p-value for the t-test of the unknown values was 0.13. After correction for the blank values and CO_2 concentrations, the average before infection was 66 (28-119). For the samples collected after infection began, the corrected average was 7,100 (1,310-13,021). The p-value for the t-test of the corrected unknown values was 0.12. The data was plotted over time for the raw and corrected unknown values by individual rat and the graphs are shown in Figures 5.14.a and b.

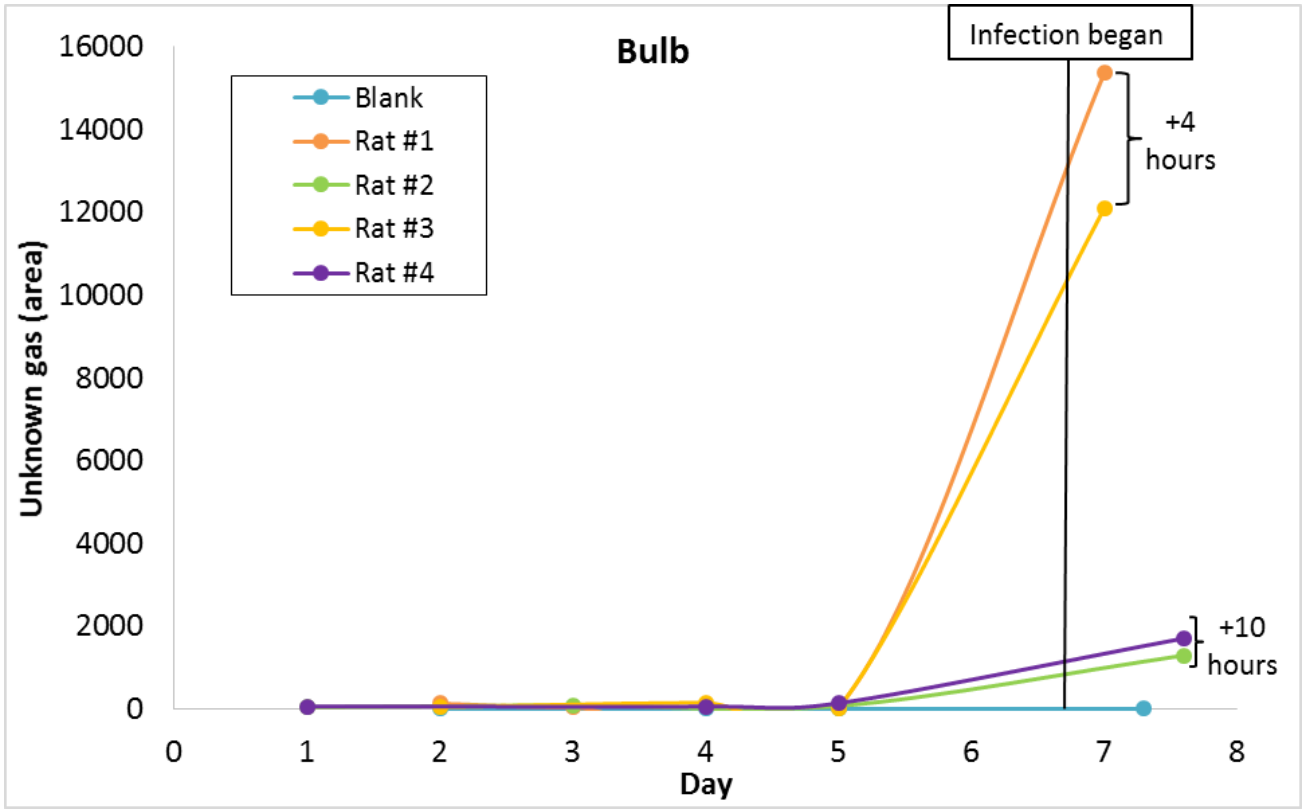

Figure 5.14.a. Plot of unknown gas over time for blank and individual rats in glass bulb.

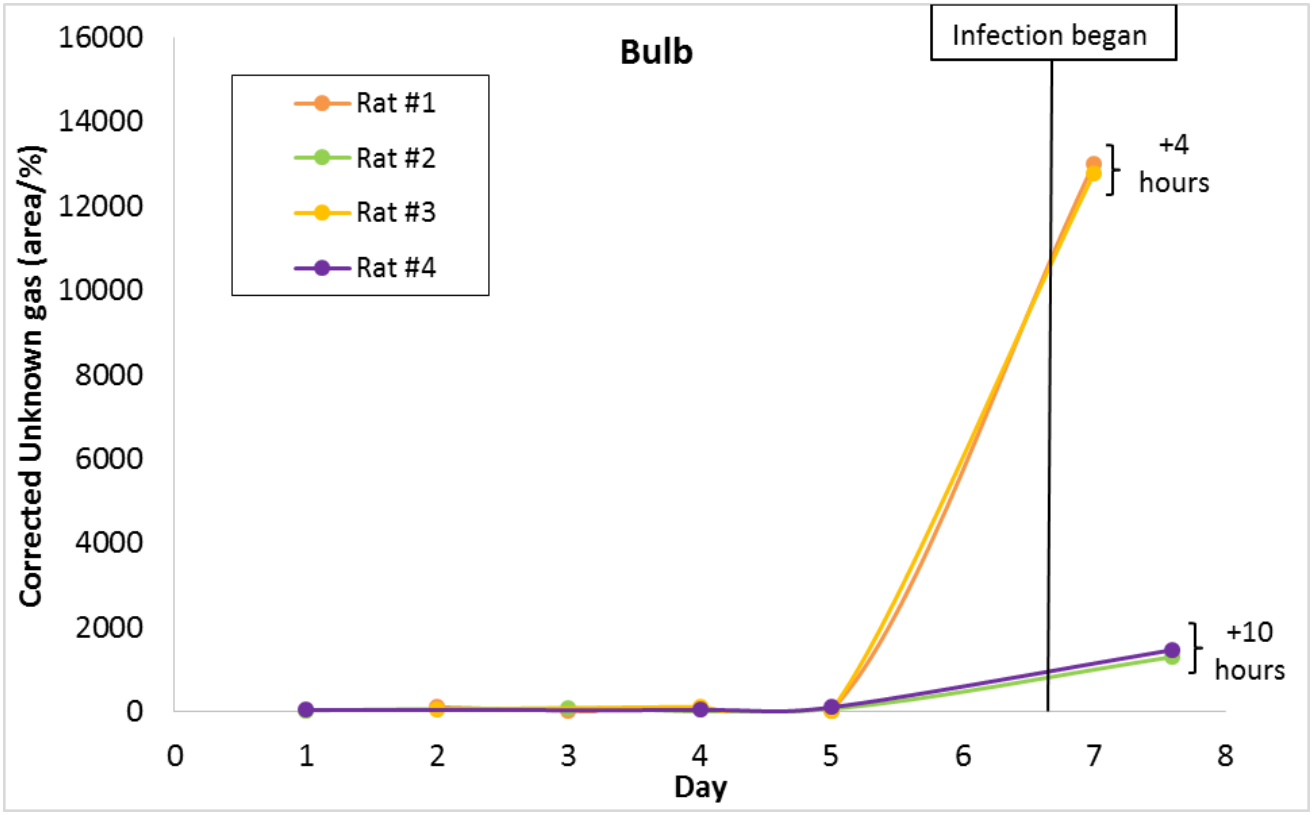

Figure 5.14.b. Plot of corrected unknown gas over time for individual rats in glass bulb.

Discussion: NMHC analysis

The NMHC analysis of the endotoxin groups and endotoxin rats study revealed a total of three gases that may be significantly different in the breath of small animals infected with LPS endotoxin. These gases were i-pentane in the case of the endotoxin groups study, acetone and a gas of unknown identity in the study performed on rats. The endotoxin rats study was performed in two different ways, the breath tower and a glass bulb, allowing for a comparison of the results. While the gases appeared to show a difference after infection in both the tower study and the glass bulb study based on averages and plots, the difference was only found to be significant by t-test analysis in the tower study. The fact that the glass bulb study had a smaller number of samples, particularly after infection began, could be the reason for the insignificant t-test finding. It is also worth noting that the acetone and unknown gas showed an opposite trend when it come to the hour and ten hour samples. In the case of the unknown, the samples taken at four hours were the highest concentrations and had returned to normal levels by ten hours. For acetone, the seven and ten hour samples were higher than the four hour samples. This could suggest that the unknown gas may have been a result of the injection itself, however because of the fact that the identity was unknown, this cannot be confirmed at this time.

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Chapter 6: Conclusion

For all pathogens discussed in this thesis, *Borrelia hermsii, salmonella enterica,* and endotoxin, the concentrations of CO and CO_2 were measured and analyzed for differences. In the salmonella study and the endotoxin study which used OSP A, there was no significant difference observed between the uninfected and infected samples for the CO concentrations nor for the corrected CO/CO_2 concentrations. For the salmonella study, this was consistent with the notion that heme oxygenase-1 production is inhibited in salmonella infections. The Borrelia and endotoxin LPS studies did show a significant increase in these concentrations between the uninfected and infected samples over time. The results of these studies could be translated into the fabrication of a handheld detector that would measure the CO and CO_2 in the breath of patients to enhance the monitoring of infections in a clinical setting.

The increase in the CO and CO/CO_2 in the Borrelia study was consistent with heme oxygenase-1 levels measured in the blood of the mice sampled. As mentioned previously, CO is a byproduct of the breakdown of heme in the blood by the heme oxygenase enzyme. In addition, in one of the studies when the mice were treated with antibiotics, the levels of CO and CO/CO_2 returned to normal levels within hours of treatment being administered. This is significant due to the fact that breath samples can be collected less invasively than traditional blood samples as well as the fact that the use of a handheld detector for breath CO/CO_2 concentration analysis would allow for immediate readout of results. This would greatly decrease the amount of wait time when compared to blood test results. The use of breath CO/CO_2 for monitoring of infections could therefore lead to a decrease in the

amount of hospitalization time as well as antibiotic use. The results of these studies suggest that breath CO and CO/CO_2 concentrations have great potential for the monitoring of infections that also increase heme oxygenase-1 levels.

The salmonella and LPS endotoxin studies were also analyzed for NMHCs and several VOCs were identified as possible markers of infection with these pathogens. In the case of salmonella, both the headspace of feces as well as the breath of mice were sampled and analyzed. The feces study identified six potential gases, OCS, DMDS, CS_2, heptanal, i-propylbenzene, and isoprene. In the breath however, there were three gases that were identified, one unknown gas, along with acetone and isopropanol. The fact that the gases found in the breath were not found to be different in the feces study suggests that these three gases do not come from the intestine where the bacteria resides. This would in turn suggest that the gases in the breath are a result of bodily processes, such as lipid peroxidation, that are affected by the infection, while the gases found in the feces study may be increased due to the presence of the salmonella bacteria.

In the LPS endotoxin studies, three gases were identified. In the study using groups of mice, i-pentane was identified while the rat study identified acetone as well as an unknown gas. Acetone was also identified as being different in the salmonella infection, suggesting it would not be a specific marker when considered on its own. It is interesting to note however that in the tower study performed with rats infected with LPS, there was one rat (#4) that did not appear to be infected based on the CO and CO/CO_2 concentrations, yet when acetone was measured, this rat was shown to have the highest concentration

after infection. This would suggest that in some cases, the use of acetone concentration paired with CO/CO_2 could increase the monitoring potential of exhaled breath.

While the use of small animals such as mice and rats can be sufficient for some breath gases, such as CO and CO_2, there are some issues that it presents. One such issue is the high amount of dilution of the breath samples which affect many of the gases that are present in lower concentrations. This effect can be decreased by using groups of mice or rats in the tower at once, however in this case individual differences in the infection status of the animals are harder to account for in this situation. In addition, the small lung volume of these animals leads to lower concentrations of many gases as well as a smaller surface for gas exchange to take place. While the data presented in this thesis can serve as a starting point for future experiments on the diagnostic and monitoring potential of exhaled breath, it is highly suggested that larger animals or even humans when possible are used. In addition, the studies presented here show the need for larger sample sizes as well as increased sample collection after infection begins.