Cold Weather Exercise and Airway Cytokine Expression

Michael S. Davis, Jerry R. Malayer, Lori Vandeventer, Christopher M. Royer, Erica C. McKenzie, and Katherine K. Williamson

Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University

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Correspondence and reprint requests should be directed to:

Michael S. Davis
Comparative Exercise Physiology Laboratory
264 McElroy Hall
Stillwater, OK 74078
405-744-8172
405-744-8263 Fax
msdavis@okstate.edu
Abstract

Athletes who perform repeated exercise while breathing cold air have a high prevalence of asthma-like chronic airway disease, but mechanism linking such activity to airway inflammation is unknown. We used a novel animal model (exercising horses) to test the hypothesis that exercise-induced chronic airway disease is caused by exposure of intrapulmonary airways to unconditioned air, resulting in the upregulation of cytokine expression. Bronchoalveolar lavage fluid (BALF) was obtained from 8 horses 5 h after submaximal exercise while breathing room temperature or subfreezing air in a random crossover design. BALF total and differential nucleated cell counts were determined and relative cytokine mRNA expression in BALF nucleated cells was quantified using real-time RT-PCR using primer and probe sequences specific for equine targets. There were no significant changes in total or differential cell concentrations between BALF recovered after warm and cold air exercise, although there was a strong trend towards increased concentrations of airway epithelial cells after cold air exercise (p = 0.0625). TH2 cytokines Interleukin (IL)-4, IL-5, and IL-10 were preferentially upregulated after cold air exercise 12, 9, and 10-fold, respectively, compared to warm air exercise. Other cytokines (IL-2 and IL-6) were upregulated to a lesser extent (6 and 3-fold, respectively) or not at all (IL-1, IL-8, Interferon-γ, and Tumor Necrosis Factor-α). These results suggest that cold weather exercise can lead to asthma-like airway disease through the local induction of cytokines typical of the TH2 phenotype.

Keywords: Exercise-induced asthma, horses, cell-mediated immunity, humoral immunity
Introduction

Exercise-induced asthma is a syndrome in which strenuous exercise, particularly while breathing cold air, can trigger airway obstruction in subjects with hyperreactive airways (31). The term “exercise-induced asthma” is something of a misnomer, as it is not the exercise itself that causes asthma in these subjects. Rather, the subjects have pre-existing airway disease, and the airway cooling and drying that occurs during periods of increased ventilation triggers reflex bronchoconstriction that spontaneously resolves within 30-60 min. However, subjects that routinely perform strenuous exercise in cold conditions have a high prevalence of chronic airway inflammation and hyperreactivity (termed “ski asthma” due to its original description in cross-country skiers (39; 40)), suggesting that such activity actually may be capable of de novo induction of an asthma-like syndrome.

We have developed an equine model of “ski asthma” to study the effects of strenuous exercise while breathing cold air on pulmonary physiology, and determine whether exercise while breathing cold air can cause chronic airway disease. Horses have greater proportional increases in cardiopulmonary function during maximal exercise compared to humans (26; 35), but in this model the exercise intensity of the horses is controlled to match typical human cardiopulmonary responses to exercise and to minimize exercise-induced pulmonary hemorrhage (a condition common in maximally-exercising horses). Previous studies with this model have confirmed the failure of the upper airways to fully warm inspired air, leading to exposure of lower airways to heat and water loss (8). These studies also confirmed that this stimulus is associated with airway mucosal damage, a feature also reported in humans after exercise while breathing cold air (37). In the current study, we used the equine model of “ski asthma” to test
the hypothesis that exercise while breathing cold air causes airway inflammation by upregulating pro-inflammatory cytokines.
Materials and Methods

All experiments were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee. Eight healthy adult horses were housed in individual stalls and fed grass hay supplemented with commercial grain mix throughout training and participation in these experiments. Training consisted of walking, trotting, and cantering 3 times weekly on a motorized high-speed treadmill in an indoor climate-controlled facility (ambient temperature 25°C) for 12 weeks. A single graded exercise test was performed after 12 weeks of training to determine the speed and slope that corresponded to a heart rate of 170 bpm for each horse, and this speed and slope were used for all subsequent exercise tests.

Horses were assigned to either cold air or warm air exercise tests in a randomized crossover design with a minimum 1 week washout between exercise tests. Each exercise test consisted of walking (1.8 m/s, 0° slope, 5 min), trotting (4 m/s, 0° slope, 5 min) and cantering (6.8-9.5 m/s, 2.5° slope, 5 min), differing only in the temperature and relative humidity of the inspired air: 25°C and 55% RH (12.6 gm H2O/m³ air) for warm air exercise tests and -5°C and >95% RH (3.5 gm H2O/m³ air) for cold air exercise tests. The cold air was produced by drawing ambient air through a high volume air chiller connected to a loose-fitting facemask worn by the horse during exercise. The volume of air delivered (3800 l/min) was in excess of the reported maximal inspiratory rate for horses exercising at submaximal levels (26) to prevent the horses from inhaling unchilled ambient air during exercise.

Five hours after completion of each exercise test, horses were sedated with intravenous xylazine (0.5 mg/kg) and butorphanol (0.025 mg/kg), and bronchoalveolar lavage was performed using a cuffed tube and 240 ml of warmed Hanks’ phosphate buffered saline. The recovered
fluid was pooled to determine overall recovery, and a 3 ml aliquot removed for determination of total and differential cell counts. Slides were prepared using cytocentrifugation and stained using a modified Wright Giemsa stain. The remaining fluid was centrifuged at 770G for 10 min, and the supernatant removed. Sixty ml of the supernatant was passed through a C-18 solid phase extraction cartridge (Water, Inc), which was subsequently eluted with 4 ml of methanol. Aliquots of the extract were evaporated to dryness and analyzed for leukotriene B4 and cysteinyl leukotrienes using commercially-available kits (Cayman Chemical, Inc). The pelleted cells were resuspended in 1 ml of a proprietary phenol/guanidine isothiocyanate mixture (Trizol, Invitrogen Inc), transferred to a 2 ml microcentrifuge tube, and homogenized using a disposable pestle. An additional 1 ml of Trizol was added to the homogenate, and the sample was frozen at -80°C until RNA extraction and cytokine message quantitation (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, and TNFα) using real time quantitative RT-PCR and species-specific primers and probes. At the time of analysis, samples were thawed and mixed with an equal volume of chloroform, and the aqueous phase was transferred to a new microcentrifuge tube. RNA was precipitated with approximately 1 ml of isopropanol, centrifuged at 13,000G, and the supernatant discarded. Precipitated RNA was washed with sequential mixing and centrifugation of ethanol and water. Total RNA was quantified spectrophotometrically based on A260/A280 ratios.

Cytokine gene expression (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, and TNFα) was evaluated by real-time quantitative RT-PCR utilizing a fluorescent reporter and 5’ exonuclease assay system (1). This technique from our previous experience is capable of efficiently amplifying and detecting a product from as few as 10 copies of the target. Reverse transcription of total RNA and PCR amplification was performed using the Taqman® One-Step RT-PCR Master Mix Reagents Kit, Taqman® fluorescent probe, and sequence detection primers
Taqman® probe specific for each target was designed to contain a fluorescent 5’ reporter dye (FAM) and 3’ quencher dye (TAMRA). Each RT-PCR reaction (15 µl) contained the following: 2X Master Mix without uracil-N-glycosylase (7.5 µl), 40X Multiscribe® and RNAse Inhibitor Mix (0.38 µl), target forward primer, target reverse primer, fluorescent labeled target probe, and sample RNA (Table 1). The PCR amplification was carried out in the ABI PRISM® 7700 Sequence Detection System (PE Biosystems). Thermal cycling conditions were 48° C for 30 minutes, 95° C for 10 minutes followed by 40 repetitive cycles of 95° C for 15 sec and 60° C for 1 minute. As a normalization control for RNA loading parallel reactions in the same multiwell plate were performed using 18S ribosomal RNA as target (18S Ribosomal Control Kit, PE Biosystems). Quantification of gene amplification was made following RT-PCR by determining the threshold cycle (C_T) number for FAM fluorescence within the geometric region of the semi-log plot generated during PCR. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. The relative quantification of target gene expression across treatments was evaluated using the comparative C_T method (1). The ∆C_T value was determined by subtracting the ribosomal C_T value for each sample from the target C_T value of that sample. Calculation of ∆ΔC_T for each cytokine and each horse involved using the ∆C_T value of the sample obtained after exercise while breathing warm air as a constant to subtract from the cold air exercise challenge values. Thus, the resulting value represents the effect of cold inspired air normalized for the effect of exercise in general. Fold changes in the relative gene expression of target was determined by evaluating the expression, 2^ΔΔCT.
Total and differential nucleated cell concentrations and leukotriene concentrations in BALF recovered after the two exercise challenges were compared using a paired Student’s t-test. Relative cytokine gene expression values were not normally distributed (Kolmogorov-Smirnov p<0.05) and therefore were analyzed using the Wilcoxon Signed Rank test using a theoretical median value of 1 (no difference from warm air or ∆∆CT = 0). In all cases, p < 0.05 was considered significant.

Results

There was no difference between warm air and cold air exercise test groups in amount of fluid recovered during bronchoalveolar lavage, nucleated cell concentration of that fluid, or total number of cells used for RNA harvesting (Table 2). There were no significant differences in the volume of recovered BALF (Warm 130.7 ± 4.7 ml; Cold 130.0 ± 6.2 ml) or concentrations of different nucleated cells in the recovered BALF (Figure 1), although there was a strong trend (p = 0.0625) towards increased bronchial epithelial cells in the lavages recovered after cold air exercise. Mast cells were detected in only half of the differential cell counts, and in most cases less than 1% of the total differential count. BALF LTB4 concentrations were not different after warm air exercise and cold air exercise (191.5 ± 97.89 vs 214.7 ± 105.6 pg/ml, respectively). BALF cysteinyll 54.38 vs 37.62 ± 20.97 pg/ml, respectively). BALF samples yielded cellular RNA of uniform consistency and with RNA concentrations exceeding 1 µg/µl in all but one sample (Table 2).

Airway cytokines characteristic of the T_{H}2 phenotype were significantly increased by exercise while breathing cold air (Figure 2). Median IL-4 mRNA expression was 12 fold greater and median IL-5 mRNA expression was 9 fold greater after exercise while breathing cold air
than after exercise while breathing warm air. Other cytokines with pro-inflammatory activity (IL-6 and IL-2) smaller, but statistically significant, increases in mRNA expression after exercise while breathing cold air (3 and 6 fold increase, respectively). The median mRNA expression of the immunomodulatory cytokine IL-10 was 10 fold greater after exercise while breathing cold air. No other measured cytokines were significantly different between exercise while breathing warm air and exercise while breathing cold air.
Discussion

Results of this study support the hypothesis that exercise while breathing cold dry air alters the airway cytokine profile. The overall cytokine pattern is predominantly a TH2 profile, which is the profile not only associated with preferential production of antibodies and downregulation of cell-mediated immunity but is also characteristic of asthma (42). However, we failed to demonstrate concurrent airway inflammation in the form of increased airway leukocytes or increased concentrations of leukotrienes. This is not necessarily surprising, since cytokine production is presumed to precede the cellular inflammatory response. Nevertheless, our results support the novel contention that exercise while breathing cold air can actually contribute to the development of asthma.

Previous studies have identified potential mechanisms for the transduction of the initial stimulus (airway cooling and desiccation secondary to exposure to unconditioned air) to airway inflammation. Both mast cells (9; 10) and airway epithelial cells (20) are osmotically-sensitive, and are activated by local airway hyperosmolarity that may occur during exercise while breathing cold air. The known osmotically-induced repertoire of cytokine production by airway epithelium is rather limited, with only IL-8 and RANTES identified in vitro (19; 20). Although we expected to find upregulation of IL-8 expression and influx of airway neutrophils, we detected neither in this study. On the other hand, mast cells are rich sources of cytokines (4; 5; 5), and although there are no studies specifically detailing cytokine production by osmotically activated mast cells, it has been previously shown that mast cells degranulate in response to hyperosmolar stimuli both in vitro (9; 10) and in vivo (18; 36). Furthermore, mast cell products are increased in blood after strenuous exercise while breathing cold air (24; 25), and drugs that
inhibit mast cells are useful in blocking exercise-induced bronchoconstriction (13-15). Other sources of cytokines, such as T-lymphocytes and macrophages, are not known to respond to non-antigenic stimuli, and are thus unlikely to be the source of initial stimulus. Thus, it is likely that mast cell activation is a key cellular event leading from airway cooling and desiccation to the expression of TH2 cytokines.

Although mast cells appear to be involved in the initial signal transduction and are capable of producing cytokines, they are unlikely to be the source of cytokine mRNA measured in this study. There were minimal numbers of mast cells recovered in the BALF from any of the horses (Figure 1). Another step leading from mast cell activation to expression of TH2 cytokines in BALF cells is needed. The BALF recovered from the horses contained ample numbers of lymphocytes, and these cells are capable of autocrine TH2 amplification after exposure to TH2 cytokines (42), even if those cytokines were initially released by other cells. Thus, we believe that peripheral airway cooling and desiccation during exercise while breathing cold air led to mast cell activation and release of TH2 cytokines, a response that was subsequently amplified by resident airway lymphocytes. Other investigators have pinpointed cellular expression of cytokine mRNA using in situ hybridization (23), and this technique would be appropriate for further studies of the equine airway responses to cold air to determine the cell populations active during these responses.

An alternative (or perhaps additional) mechanism for transducing the initial airway stimulus to preferential secretion of TH2 cytokines is through the release of neurokinins. Neuronkinins have been shown to induce secretion of some TH2 cytokines from T-lymphocytes, even when those lymphocytes have been committed to a TH1 phenotype (27), and selective
inhibition of NK-2 receptors can block the development of eosinophilia and expression of \( T_{H2} \) cytokines after allergen challenge in allergic mice (30). Neurokinins are released into the airway following hyperpnea challenge in a number of animal models, either as a primary event or secondary to leukotriene release (17). If the latter is true, then the lack of detectable change in BALF leukotrienes argues against a role for neurokinins in the induction of \( T_{H2} \) cytokines in equine airways after cold weather exercise. However, more direct studies are required to confirm or exclude this phenomenon.

A limitation of this study is that although we have described the effect of inspiring cold air while exercising, we have no information regarding the effect of the exercise challenge itself. Suzuki et al has shown that the circulating lymphocyte population is biased towards \( T_{H2} \) profile of cytokine secretion following exercise (41), an effect that may be due to concurrent release of glucocorticoids during exercise (11; 12). A similar pattern has been described in mice (22). These findings raise the question of whether the pattern of cytokine production in the airway cells is simply a reflection of systemic predisposition towards a \( T_{H2} \) phenotype, secondary to exercise induced cortisol secretion. Although we can not completely refute this possibility, it is interesting to note that Ainsworth et al failed to demonstrate a shift in cytokine expression in peripheral blood following exercise in horses (2). Furthermore, we found no significant difference in circulating cortisol concentrations in a different group of horses performing similar exercise while breathing warm and cold air (unpublished data). These observations, combined with the tenable hypothesis for upregulation of cytokine expression following cold air exercise, support our contention that the findings described in this paper are caused by local airway conditions or events.
The potential effect of the cytokine expression shift described in this study and the resulting alteration in antigen responses is consistent with common pulmonary diseases of both equine and human athletes. Antibody-mediated pulmonary hypersensitivity (“heaves”) is common in horses (3), and has been linked to increased expression of \( \text{T}_{\text{H}2} \) cytokine expression (6; 23). The most prominent antigens identified in this syndrome are mold spores found in hay and straw bedding (32; 33), thus closely linking the development of heaves to the horse’s environment. Once a horse has developed such hypersensitivity, clinical exacerbations consisting of airway inflammation, recurrent airway obstruction, and airway mechanical hyperreactivity can be readily reproduced with environmental exposure to specific allergens, and remission can be achieved upon removal of the horse from an environment conducive to allergen exposure (38). In this regard, heaves is quite similar to human asthma, including the fact that the initial cause of the hypersensitivity has not been elucidated. The data of this study provide a compelling possibility: that strenuous exercise followed by exposure to environmental antigens promotes overproduction of antibodies to those antigens. It would follow from this line of reasoning that heaves is more prevalent in athletic horses compared to sedentary cohorts, and that heaves is more prevalent in colder climates compared to warmer climates. Unfortunately, neither of these possibilities has been critically explored in the existing literature.

The implications of these data can be extended to the syndrome of “ski asthma”. The precise nature of the inflammation characteristic of “ski asthma”, including the relative cytokine expression in the airways of affected subjects, has not been described. However, increased numbers of eosinophils in the airways of subjects with ski asthma (21) strongly implicates increased expression of IL-5, one of the hallmark cytokines of the \( \text{T}_{\text{H}2} \) phenotype (28) and one of the cytokines that were significantly expressed after exercise while breathing cold air (Figure 2).
Thus, our data not only supports the contention that some, if not all, of the abnormalities described for ski asthma result from exposure of airways to unconditioned air, but also raises the question of whether other T_{h}2-associated cytokines are increased in these subjects.

Our data further raises the possibility of local suppression of cell-mediated immunity through the increased expression of IL-10. The concept of exercise-induced immunosuppression is not novel, as many investigators have produced data supporting the concept of the “open window” of transient immune suppression following strenuous exercise (34). Furthermore, previous studies have demonstrated increased susceptibility to respiratory viruses in animals after strenuous exercise (7; 16). However, we believe our data are the first to provide a specific mechanism for the exercise-induced open window effect as a local pulmonary phenomenon. The potential deleterious effect of increased IL-10 expression is increased susceptibility to pathogens, particularly those that are normally cleared by cell-mediated immunity (29). On the other hand, it is possible that the increase in IL-10 is an appropriate response that moderates the net effect of the increased pro-inflammatory cytokine expression. These issues warrant further investigation, for which the equine model is ideally suited.
Text Footnotes
Acknowledgments
Grants

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    prostanoids to bronchoconstriction provoked by isocapnic hyperventilation in


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induced interleukin-8 and RANTES production by human bronchial epithelial cells.


**Figure Legends**

Figure 1: Airway Nucleated Cells after Exercise while Breathing Cold Air. Total Cells – Total BALF Nucleated Cell Concentration; Macro - Macrophages; Lymph - Lymphocytes; PMN - Neutrophils; Eos - Eosinophils; Epith - Epithelial cells. PMN, Eos, and Epith are plotted against the right Y-axis. Mast cells were rarely found during differential cell counts, and accounted for less than 1% of the overall cells in both groups.

Figure 2: Airway Cytokine mRNA Expression after Exercise while Breathing Cold Air. Data are expressed as the relative (fold) increase of cytokine expression after cold air exercise compared to warm air exercise. *Significantly different from 1 (relative expression after exercise while breathing warm air), p < 0.05.
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Tables

Table 1. Cytokines quantified in the study, primer/probe sequences, primer/probe reaction concentrations, total RNA used to quantify \( \Delta \Delta C_T \) values.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>Primer/Probe (nM)</th>
<th>Total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1 α</td>
<td>TGGGAACGTACGGCTCTA</td>
<td>CCTGCTTTGGCAATAAAC</td>
<td>ACTACCTCAAATCGGTTGCCCATCAAAGT</td>
<td>900/900</td>
<td>100 ng</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>CTGCAGCGGCAATGAGAAT</td>
<td>TGGAAAGCTGCCCTTCATCTG</td>
<td>TGGGCCATCTCTCAAAGAACAGGT</td>
<td>700/700</td>
<td>50 ng</td>
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<td>IL-2</td>
<td>TGGCTTGATCGCACAAA</td>
<td>TGTTTGGTGTGGTCTCCTAGAG</td>
<td>CAGTCCTDGAAACAGTGACCTACTTCAAA</td>
<td>900/900</td>
<td>100 ng</td>
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<tr>
<td>IL-4</td>
<td>CGTCATGGGAGTACGGTACG</td>
<td>CAGCCCTGCAGATTTCTTT</td>
<td>ATGCCCTTGCCGAAAGAACAGA</td>
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<td>50 ng</td>
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<tr>
<td>IL-5</td>
<td>TGCTCAGGTGAGTTAGGTTT</td>
<td>TGTTGAGCTGCTACGTCTGACCA</td>
<td>CTCTTTGAGCCCTCCGTGGGAG</td>
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<td>50 ng</td>
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<tr>
<td>IL-6</td>
<td>TGCTAAGGCTGCATCCAAG</td>
<td>GGAATCCCTCAAAGCTCAG</td>
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<td>900/900</td>
<td>50 ng</td>
</tr>
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<td>IL-8</td>
<td>CTTCAAGGCTGGCTGACT</td>
<td>CAAACGGAGCTCTCACAAGA</td>
<td>CTGAGCGCTTTATCTTGCTCTGCA</td>
<td>900/900</td>
<td>50 ng</td>
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<tr>
<td>IL-10</td>
<td>GATCTCCAAAATCCCATCCA</td>
<td>AGGAGAGAGGTACCCACAGGTTT</td>
<td>CCAAGGAGCTTGATTCAGCTCTTCCAGA</td>
<td>700/700</td>
<td>50 ng</td>
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<tr>
<td>IFN γ</td>
<td>AATGGAACACTCAATCAAAGTAGGAAGTGA</td>
<td>GAAATGGGATCTGACTTCCTTC</td>
<td>TGGCCAAAGCTAAGCTGAGAAGC</td>
<td>700/700</td>
<td>50 ng</td>
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<tr>
<td>TNF α</td>
<td>GATGACCTTGCTGATGCTAATCC</td>
<td>TCTGGGGCCAGAGGTTGAT</td>
<td>TCCCCAGCAGTATCCGATGCTCTC</td>
<td>900/900</td>
<td>100 ng</td>
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Table 2: Purity and concentration of RNA used to evaluate airway cytokine expression.

<table>
<thead>
<tr>
<th>Horse Sample</th>
<th>$A_{260}$</th>
<th>$A_{280}$</th>
<th>$A_{260}/A_{280}$</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Bella: Warm Air</td>
<td>0.552</td>
<td>0.290</td>
<td>1.9</td>
<td>1.1 µg/µl</td>
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<tr>
<td>Bella: Cold Air</td>
<td>0.804</td>
<td>0.419</td>
<td>1.9</td>
<td>1.61 µg/µl</td>
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<td>Blondie: Warm Air</td>
<td>0.609</td>
<td>0.319</td>
<td>1.9</td>
<td>2.44 µg/µl</td>
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<tr>
<td>Blondie: Cold Air</td>
<td>0.608</td>
<td>0.322</td>
<td>1.9</td>
<td>2.43 µg/µl</td>
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<tr>
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<td>0.919</td>
<td>0.516</td>
<td>1.8</td>
<td>3.68 µg/µl</td>
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<tr>
<td>Coalie: Cold Air</td>
<td>0.430</td>
<td>0.267</td>
<td>1.7</td>
<td>1.72 µg/µl</td>
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<tr>
<td>Ivan: Warm Air</td>
<td>0.914</td>
<td>4.78</td>
<td>1.9</td>
<td>1.83 µg/µl</td>
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<td>Ivan: Cold Air</td>
<td>0.690</td>
<td>0.387</td>
<td>1.8</td>
<td>1.38 µg/µl</td>
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<td>Major: Warm Air</td>
<td>0.289</td>
<td>0.177</td>
<td>1.7</td>
<td>1.156 µg/µl</td>
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<tr>
<td>Major: Cold Air</td>
<td>0.722</td>
<td>0.382</td>
<td>1.9</td>
<td>2.89 µg/µl</td>
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<td>Poco: Warm Air</td>
<td>0.736</td>
<td>0.386</td>
<td>1.9</td>
<td>2.94 µg/µl</td>
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<td>Poco: Cold Air</td>
<td>0.714</td>
<td>0.371</td>
<td>1.9</td>
<td>1.43 µg/µl</td>
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<tr>
<td>Skipper: Warm Air</td>
<td>0.585</td>
<td>0.304</td>
<td>1.9</td>
<td>2.34 µg/µl</td>
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<td>Skipper: Cold Air</td>
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<td>0.378</td>
<td>1.9</td>
<td>1.4 µg/µl</td>
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<td>Xena: Warm Air</td>
<td>0.452</td>
<td>0.269</td>
<td>1.7</td>
<td>0.904 µg/µl</td>
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<td>Xena: Cold Air</td>
<td>0.706</td>
<td>0.375</td>
<td>1.9</td>
<td>1.41 µg/µl</td>
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