

University of Nevada, Reno

**Effects of Hypoxia and High Altitude on  
Gene Expression, Energetics, and Immune Function**

A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy in Ecology, Evolution, and Conservation Biology

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prepared under our supervision by

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**ABSTRACT**

Environmental stressors, which shape the evolution and ecology of a species, are of major interest to physiological ecologists. The hypoxia encountered at high altitude is a well known abiotic stressor, and how animals cope has long intrigued both physiologists and ecologists. Despite the long interest in high altitude physiology, researchers are continuing to make discoveries in how mammals physiologically acclimatize and adapt to the chronic hypoxia found at high altitude. To add to this growing knowledge, DNA microarray-based mRNA expression profiling was used to investigate responses to hypoxia. In the first chapter, C57BL/6 strain laboratory mice (*Mus domesticus*) were subjected to 32 days of hypoxia and liver samples were used for analysis of global gene expression patterns. ANOVA methods identified 580 genes that were statistically significantly differentially expressed in response to chronic hypoxia. Few of these 580 genes had previously been reported to respond to hypoxia. However, many of the 580 genes belonged to functional groups that are important for responding to acute hypoxia (e.g., angiogenesis, glycolysis, lipid metabolism, carbohydrate metabolism, and protein amino acid phosphorylation). Novel to this study were the increased expression of leptin receptor and the differential expression of genes associated with the immune system.

In the second chapter, DNA microarray-based mRNA expression profiling was used to compare gene expression of wild populations of house mice (*Mus musculus domesticus*) living at high and low altitudes. One way and nested ANOVA analyses identified 107 statistically significantly differentially expressed genes, few of which were in common with the laboratory study in the first chapter. When compared with mice at low altitude, mice at high altitude differentially expressed genes with functions associated with the immune system. This effect of altitude on expression of immune genes is consistent with the differential expression of immune genes in hypoxic mice found in the first chapter. Furthermore, EGLN3, a gene responsible for the negative regulation of Hypoxia

Inducible Factor (HIF), had decreased expression in high altitude mice. EGLN3 is a homolog to EGLN1 that was recently identified as a gene responsible for evolutionary adaptation in high altitude humans.

The third chapter follows up on the differential expression of immune system genes in both chronic hypoxia and high altitude. While there is growing evidence that acute hypoxia has proinflammatory effects, it is unknown whether or not chronic hypoxia would have the same effect. At least some studies by ecological immunologists have suggested that immune function may be energetically costly. If hypoxia imposes an energetic stress, then an energetic tradeoff hypothesis would predict that the putatively costly immune system should not be up-regulated during this hypoxic stress. I tested (i) whether or not chronic hypoxia affects immune function, and (ii) whether or not hypoxia affects the metabolic cost of immune function. First, flow cytometry was used to monitor the peripheral blood immunophenotype of mice over the course of 36 days of hypoxic exposure. Second, hypoxic and normoxic mice were subjected to an adaptive immune challenge via keyhole limpet hemocyanin (KLH), or to an innate immune challenge via lipopolysaccharide (LPS). The resting metabolic rates of mice in all immune challenge treatments were also measured. Although hypoxia had little effect on the peripheral blood immunophenotype, hypoxic mice challenged with KLH or LPS had enhanced immunological responses in the form of either higher antibody titers or increased TNF- $\alpha$  production, respectively. Initially mice exposed to hypoxia had lower metabolic rates, but this response was transitory and resting metabolic rates were normal by the end of the experiment. Surprisingly, there was no effect of either immune challenge on resting metabolic rate, suggesting that mounting either the acute phase response or a humoral response is not as energetically expensive as previously thought. Taken together, these data indicate that hypoxia has a positive effect on the immune system independent of an energetic relationship. Therefore we propose that the relationship between the immune system and hypoxic stress is governed not by

demand for metabolic energy, but by shared transcriptional and hormonal networks that regulate both processes. Further research into the relationship between hypoxic stress and the immune system will help elucidate how the immune system is sensitive to environmental stresses, and how the immune system may be important to the physiological ecology of mammals at high altitude.

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## INTRODUCTION

Understanding how organisms cope with, acclimate to, and eventually adapt to environmental stress is an important and central topic in ecology and evolutionary biology. While abiotic stressors are important forces in physiological evolution, much remains to be learned about evolutionary mechanisms by which animals acclimate, acclimatize, and adapt to these stresses encountered in the environment (Badyaev, 2005; Lexer and Fay, 2005). One major mechanism underlying physiological change is the regulation of genes and their products (Schlichting and Smith, 2002). Hence, physiologists are increasingly utilizing genomic tools to investigate physiological change. In particular, high throughput studies of steady-state mRNA expression with DNA microarrays have proven useful in generating hypotheses concerning the mechanisms of acclimatization and adaptation (Gracey, 2007; Gracey and Cossins, 2003).

Hypoxia (i.e., the reduced availability of oxygen) is a useful model of environmental stress for better understanding these transcriptomic mechanisms of acclimation, acclimatization and adaptation. Acclimatization and adaptation of mammals to high altitude hypoxia have received much attention due to their biomedical and ecological relevance (Hochachka and Somero, 2002; Julian et al., 2009; Powell, 2003; Raguso et al., 2004; Ramirez et al., 1999; Ramirez et al., 2007; Rhodes, 2005; Storz and Moriyama, 2008; Storz et al., 2009; Ward et al., 2000). The physiological responses of mammals to chronic hypoxia are well described, and include increased hemoglobin production (polycythemia), increased production of 2,3 bisphosphoglycerate, an allosteric effector of hemoglobin-oxygen binding affinity, pulmonary vasoconstriction, increased lung and liver mass; increased left ventricular mass, increased ventilation rate and tidal volume, increased capillary density (angiogenesis), and anorexia with subsequent weight loss (Appenzeller et al., 2003; Hammond et al., 2001; Ward et al., 2000). In addition, while the above physiological changes are taking place, many mammals alter their

metabolism to decrease their demand for oxygen by increasing anaerobic glycolysis and glucose utilization, decreasing whole-animal metabolic rate, and decreasing body temperature (Gautier, 1996; Hochachka et al., 1996; Semenza et al., 1994; Steiner and Branco, 2002).

While much is known about the physiological acclimatization to chronic or high altitude hypoxia, few studies have focused on the transcriptomic or proteomic changes that underpin acclimatization. Most studies of the effects of hypoxia on gene and protein expression have focused on responses of cells to acute hypoxia (seconds to hours of exposure). Acute responses to cellular hypoxia include changes in a number of transcription factors that are essential for regulating cell development, apoptosis, cell proliferation and differentiation, and inflammation (Cummins and Taylor, 2005; Kenneth and Rocha, 2008). Hypoxia inducible factor (HIF) is the best understood of these transcription factors. Post-translation modification of HIF during hypoxia leads to the transcription of more than 70 hypoxia responsive genes. Many of these genes are associated with physiological changes observed in response to chronic hypoxia (Sarkar et al., 2003). For example, in response to hypoxia, HIF causes up-regulation of (a) erythropoietin (EPO), which is involved in erythropoiesis (increasing red blood cell production), (b) transferrin, a key to iron transport and metabolism, (c) genes associated with angiogenesis, such as vascular endothelial growth factor (VEGF), and (d) genes associated with many metabolic substrates, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pyruvate kinase, which increase glucose uptake and anaerobic glycolysis (Bracken et al., 2003; Semenza, 2000). Indeed, a deficiency of HIF-1 $\alpha$ , one of the HIF subunits, dramatically inhibits long-term physiological responses to hypoxia (Yu et al., 1999). Thus, the HIF pathway plays an important role in both acute responses and physiological acclimation to hypoxia. However, whether or not these patterns of gene expression seen during acute hypoxia are similar to those during chronic hypoxia remains unexplored.

Many studies investigating genomic responses to hypoxia are limited in that they are often conducted using *in vitro* cell lines, or that they focus on responses to very acute exposures to hypoxia. Because of this, the understanding of how gene expression is changed during chronic hypoxia, or during a lifetime of hypoxia at high altitude, is limited. The first two chapters of this dissertation investigate (1) the effects of chronic hypoxia on global gene expression patterns, and (2) the natural variation in gene expression that is present between high and low altitude populations. Both experiments utilize the Affymetrix GeneChip<sup>®</sup> Mouse Genome 430 2.0 Array to investigate differences in gene expression for the approximately 40,000 genes known to be expressed in the mouse genome. The first chapter explores the transcriptomic responses of inbred C57BL/6 mice that have acclimated to hypoxia for 32 days. Because responses to acute, cellular hypoxia may be different from responses to chronic, systemic hypoxia, the genes that are responsible for maintaining an acclimatized state may be quite different from the genes that initially respond to a decrease in oxygen availability. My first chapter, which was published in the May 2010 issue of *Physiological Genomics* (Baze et al., 2010), identified novel gene expression responses to hypoxia, and data from this experiment were used to generate hypotheses regarding physiological changes in response to hypoxia that may not have previously been appreciated. In particular, probesets representing leptin receptor,  $\alpha$ -globin, and  $\beta$ -globin all reported significant increases in expression in response to hypoxia, indicating that expression of these genes might be important for maintaining hypoxia acclimation. Furthermore, genes related to the immune system showed differential expression, suggesting that the immune system might be important in hypoxia acclimation.

In my second chapter, we used DNA microarrays to compare gene expression in wild populations of house mice (*Mus musculus domesticus*) living in the high altitude city of La Paz, Bolivia (3000-3800 m) and living in the low altitude city of Lima, Peru (0-200 m). While one

might expect that some of the genes that support acclimatization to hypoxia may also be differentially expressed in animals residing permanently at high altitude, this hypothesis was not supported. However, this study identified EGL homolog 3 as a potentially important gene at high altitude. This result is consistent with recent findings that EGLN1 polymorphisms and decreased expression are associated with high altitude adaptation in humans. Furthermore, like laboratory mice acclimated to hypoxia, wild mice residing at high altitude also showed differential expression of genes related to the immune system.

The first two chapters both identified significant differences in the expression of genes related to the immune system. The immune system is not usually considered to be important when responding to hypoxia. Yet, there is growing evidence that acute, cellular hypoxia has a pro-inflammatory effect on macrophages, neutrophils and other leukocytes, and an anti-inflammatory effect on some lymphocytes (Bosco et al., 2006; Conforti et al., 2003; Kojima et al., 2002; Murdoch et al., 2005; Thake et al., 2004; Walmsley et al., 2005). Furthermore, some studies report that humans who travel to high altitude environments have increased circulating leukocytes and decreased circulating CD4 T-cells (Chohan and Singh, 1979; Chohan et al., 1975; Facco et al., 2005). Why such an immune response should occur during hypoxia or high altitude, and of what benefit it could be, is not well known.

The third chapter follows up on the findings that the immune system may be important during acclimation to hypoxia and life at high altitude. I explored the relationship between the immune system and hypoxia acclimation from the perspective of ecological immunology. Ecological immunologists seek to understand how variation in the immune function is related to organismal fitness within different environmental and ecological conditions. A major aspect of ecological immunology is in understanding the potential tradeoffs between immune functions and life history traits (Lochmiller and Deerenberg, 2000; Schmid-Hempel and Ebert, 2003; Sheldon

and Verhulst, 1996; Svensson, 1997). A widespread idea in ecological immunology is that the immune system is energetically expensive, and an induction or a significant investment in the immune system will reduce the energy available for other physiological processes (Lochmiller and Deerenberg 2000). Attempts to find empirical evidence for this have turned up conflicting results (Lee, 2006; Norris and Evans, 2000).

From the energetic tradeoff prediction, one would not predict an increased inflammatory response to hypoxia. Coping with hypoxia leads to a number of potential energetic challenges in small mammals. For instance, there are potential increased energetic costs associated with polycythemia, increasing organ mass, and increasing vascularization. Furthermore, small mammals often decrease their body temperature and metabolic rates in response to hypoxic stress. (Frappell et al., 1992; Gautier, 1996). Thus, assuming that the immune system is energetically costly, an induction of the immune system is contrary to the expectations set forth by the energetic tradeoff hypothesis of ecological immunology.

In my third chapter, I first test the hypothesis that hypoxia alone affects the makeup of the peripheral immune system. To accomplish this, flow cytometry was used to profile the blood of inbred mice housed in hypoxic and normoxic laboratory environments. I also investigated the energetic costs of acclimating to hypoxia, of mounting both adaptive and innate immune responses, and of responding to both hypoxia and immune challenges simultaneously. By assessing the simultaneous effects of hypoxia and immune function, I test the hypothesis that physiological acclimation to hypoxia and response to immune challenges will lead to competition for metabolic energy.

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**CHAPTER 1: GENE EXPRESSION OF THE LIVER IN RESPONSE TO CHRONIC HYPOXIA.**

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**ABSTRACT**

Hypoxia is an important ecological, evolutionary, and biomedical stressor. While physiological acclimatization of mammals to hypoxia and high altitudes is well studied, the variation in gene expression that underlies acclimatization is not well studied. We acclimatized inbred mice for 32 days to hypoxic conditions that simulated altitudes of 1400 m, 3000 m and 4500 m. We used oligonucleotide microarrays to measure changes in steady-state abundance of mRNA in the livers of these mice. Mice exposed to more severe hypoxia (simulated altitude of 4500 m) were smaller in mass and had higher hematocrit than mice exposed to less severe hypoxia. ANOVA and false discovery rate tests indicated that 580 genes were significantly differentially expressed in response to chronic hypoxia. Few of these 580 genes have previously been reported to respond to hypoxia. In contrast, many of these 580 genes belonged to same functional groups typically respond to acute hypoxia. That is, both chronic and acute hypoxia elicit changes in transcript abundance for genes involved in angiogenesis, glycolysis, lipid metabolism, carbohydrate metabolism, and protein amino acid phosphorylation, but the particular genes affected by the two types of hypoxia were mostly different. The transcript showing the most elevated response to chronic hypoxia was leptin receptor, suggesting that the leptin signaling pathway is an important feature of acclimation to hypoxia. Numerous genes affecting the immune system were differentially expressed in response to chronic hypoxia, which supports recently proposed hypothetical links between immune function and hypoxia. Genes involved in hematopoiesis and oxygen transport were also upregulated in response to severe chronic hypoxia, which is peculiar for liver tissue. These data, in concert with the extremely high hematocrits (80.4%) for mice exposed to that altitude, suggest the possibility that the liver may engage in extramedullary hematopoiesis in response to severe, chronic hypoxic stress.

## INTRODUCTION

The survival of an organism often depends on its ability to acclimatize to environmental stressors. Acclimatization results from remodeling of the organism's physiological and anatomical phenotype in an attempt to accommodate an environmental stressor. A major mechanism underlying physiological remodeling is the regulation of genes and their products (84). Hence, physiologists are increasingly utilizing genomic tools to investigate physiological change. In particular, high throughput studies of steady-state mRNA expression with DNA microarrays have proven useful in generating hypotheses concerning the mechanisms of acclimatization (34, 35).

Acclimatization and adaptation of mammals to high altitude hypoxia (reduced partial pressure of oxygen ( $pO_2$ )) has received much attention due to its biomedical and ecological relevance (46, 51, 70, 72-74, 79, 92, 93, 103). Mammals experiencing chronic hypoxia undertake diverse responses aimed at maintaining oxygen delivery despite a reduced driving force (partial pressure gradient) for delivering oxygen. These responses include: increased hemoglobin production with a concomitant increase in hematocrit and blood oxygen carrying capacity; increased production of 2,3 bisphosphoglycerate, an allosteric effector of hemoglobin-oxygen binding affinity; pulmonary vasoconstriction; increased lung and liver mass; increased left ventricular mass, which leads to increased stroke volume; increased tidal volume and ventilation rate; increased capillary density; and anorexia and subsequent weight loss (1, 6, 12, 33, 64, 87). In addition, while the above physiological changes are taking place, many mammals alter their metabolism to decrease their demand for oxygen. These metabolic alterations can include increased anaerobic glycolysis and glucose utilization, decreased whole-animal metabolic rate, and decreased body temperature (32, 45, 86, 90).

While much is known about the physiological acclimatization to high altitude hypoxia, few studies have focused on the underlying molecular genetic changes. Most studies of the effects of hypoxia on gene and protein expression have focused on responses of cells to acute hypoxia (seconds to hours of exposure). Acute responses to cellular hypoxia include changes in a number of transcription factors, such as AP-1, NF- $\kappa$ B, p53, Erg-1, and the Myc family of proteins. These transcription factors respond to a variety of stressors, and they are essential for regulating cell development, apoptosis, cell proliferation and differentiation, and inflammation (20, 54). Hypoxia inducible factor (HIF) is the best understood of the transcription factors that responds to hypoxia. HIF is a heterodimer of HIF-1 $\beta$  and one of three tissue specific, hypoxia regulated dimers, HIF-1 $\alpha$ , HIF-2 $\alpha$ , or HIF-3 $\alpha$ . The HIF- $\alpha$  subunits rapidly accumulate during hypoxic conditions, dimerize with HIF-1 $\beta$  and induce the transcription of more than 70 hypoxia responsive genes (40, 42, 52). Many of these genes are associated with physiological changes observed in response to hypoxia (81). For example, in response to hypoxia, HIF causes upregulation of (a) erythropoietin (EPO), which is involved in erythropoiesis (increasing red blood cell production), (b) transferrin, a key to iron transport and metabolism, (c) genes associated with angiogenesis, such as vascular endothelial growth factor (VEGF), and (d) genes associated with many metabolic substrates, such as GAPDH and pyruvate kinase, which increase glucose uptake and anaerobic glycolysis (11, 85). Indeed, a deficiency of HIF-1 $\alpha$ , one of the HIF subunits, dramatically inhibits long-term physiological responses to hypoxia (107). Thus, the HIF pathway plays an important role in both acute responses and physiological acclimation to hypoxia. However, the relationship between acute and chronic hypoxic responses remains unexplored.

A limitation of many studies investigating genomic responses to hypoxia is that they are often conducted using *in vitro* cell lines. Focusing on the cellular response to hypoxia removes

the cells from their normal environment of the tissue within the organism. In recent years, increased attention has been placed on the effect of hypoxia on tissues *in vivo* (21, 25, 47). From the few studies that have employed multiple *in vivo* tissues, it appears that changes in gene expression are likely to vary with how the tissues experience hypoxia and with their metabolic role within the organism (8, 36, 80, 94, 105). Whereas previous studies have focused primarily on acute or intermittent hypoxia, none have compared genome-wide gene expression of mammals acclimated to constant hypoxia for extended periods of time (i.e., weeks) with unacclimated controls.

Herein we document changes in steady-state mRNA expression in the livers of house mice acclimated to normobaric hypoxia for 32 days. Much of what we know of the cellular response to hypoxia was established with hepatocyte cell lines (4, 27, 33, 86, 102), and the liver is known to alter gene expression profile in response to acute hypoxia *in vivo* (21, 52). The response of the liver to hypoxia is not surprising given that the liver plays a central role in regulating energy balance, substrate metabolism, and detoxification, and a small role in erythropoiesis (23). All of these processes are affected by hypoxia, and they may be critical for acclimatizing to hypoxia. For example, acute hypoxia stimulates cells to switch from oxidative phosphorylation to anaerobic glycolysis, while perhaps decreasing fatty acid utilization. Increased gluconeogenesis performed by the liver can supply these hypoxic tissues with the glucose that they require (16) while increasing lipid storage of circulating fatty acids (13, 76). In contrast, during chronic hypoxia liver substrate preference appears to switch from glucose and anaerobic glycolysis towards fatty acid metabolism (68). Under normal circumstances, the liver

produces small amounts of EPO compared to the kidney, but under extreme hypoxia, production of EPO by the liver increases substantially (75). Patients suffering from COPD have decreased drug clearance ability, and this response has been linked to the decreased expression of various Cytochrome P450 isoforms by the hypoxic liver (28). Finally, the liver is one of the few tissues that expresses all three HIF isoforms (HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ ), which suggests that it is important in responding to hypoxic conditions (76). Because the liver is a key mediator of metabolic process and because it plays an important role in acclimatization to hypoxic, we focused our study on the liver (52).

## MATERIALS AND METHODS

### *Treatments and Animal Handling.*

We obtained 36 male mice of the inbred strain C57BL/6 from Charles River Laboratories (Hollister, CA). The C57BL/6 strain for mice was chosen because this is the model strain for which genome sequencing and subsequent array designs have been based on. After acclimating to our vivarium in Reno, Nevada for one week, the 10 week old mice were randomly assigned to one of three simulated altitude treatments. The treatments approximated the oxygen partial pressures found at 1400 m (18.0 kPa), 3000 m (14.5 kPa) and 4500 m (11.5 kPa). The oxygen partial pressures were achieved by adding appropriate amounts of nitrogen to air using thermal mass flow controllers, and supplying this mixed air to each of three 150 L environmental chambers. Each treatment consisted of one chamber with 12 mice. Reno, NV, USA is located at 1400 m above sea level and the ambient barometric pressures averages 86.1 kPa. The 1400 m treatment received ambient air. The 3000 m group received air with 15.3% oxygen, and the 4500 m group received air with 11.8% oxygen. Because animals inside the environmental chamber consume oxygen, the concentration of oxygen leaving the environmental chambers (i.e., the excurrent concentration) will be lower than the concentration entering the chamber (incurrent concentration). The faster the flow rate through the chamber is, the lower the oxygen concentration difference will be between the inlet and outlet air. Hence, faster flowrates are good for minimizing the oxygen concentration difference but they also require more nitrogen and more frequent changing of the gas cylinders providing the nitrogen. As a compromise between these two constraints, we calculated the flowrate necessary to keep the incurrent and excurrent concentration within 1% O<sub>2</sub> using the formula  $VO_2 = FR_i (O_2C_i - O_2C_e) / (1 - O_2C_e)$ , where  $VO_2$  = oxygen consumed,  $FR$  = flowrate,  $O_2C$  = fractional oxygen concentration (e.g.,  $O_2C$  for ambient air  $\approx 0.2095$ ), the subscripts  $i$  and  $e$  stands for incurrent and excurrent, respectively, (106). To be

conservative, we estimated total  $\text{VO}_2$  in the chamber as 24 times the average daily oxygen consumption reported by Jackson Laboratories (Bar Harbor, Maine, [www.jax.org](http://www.jax.org)) for adult male C57BL/6 mice. That is, we calculated the flowrate needed to keep the incurrent and excurrent oxygen concentrations within 1%  $\text{O}_2$  even if the oxygen consumption of the 12 mice per chamber was double the normal oxygen consumption. Based on this calculation, we estimated that a flowrate of  $\sim 2.5$  L/min was needed, although the flow delivered to each chamber ranged from 2.5 L/min to 2.8 L/min. Periodic checks over the course of the experiment indicated that the average oxygen concentrations of excurrent air were 20.2, 14.5, and 11.2% for the 1400m, 3000m and 4500m treatments, respectively. These values compare with average incurrent concentrations of 20.9%, 15.3, and 11.8%, respectively. We acknowledge that imperfect mixing may have resulted in some variation in  $\text{pO}_2$  within each chamber and that the slight pressure differential needed to ensure airflow through each chamber may have resulted in achieved  $\text{pO}_2$ 's that were slightly different than those that would be calculated from our data on oxygen concentration and ambient barometric pressure.

Within each 150 L chamber, the 12 mice were individually housed in 30 cm x 12 cm x 8 cm standard rodent cages and provided with food and water *ad libitum* for 32 days. The chambers were maintained in a room with 12L:12D cycle and at approximately 21 °C (i.e., room temperature, which reflected ambient building condition, not a precisely regulated thermal environment). Data from other related experiments indicates that temperatures within the chamber did not vary significantly from the ambient temperature of the room. The chambers were opened briefly on days 9, 17, 24, and 30 to change cages, add food and water and weigh the mice.

After 32 days, two days after the final body mass measurements, we removed the mice, took two blood samples from the infraorbital sinus with microhematocrit tubes and sacrificed the mice via cervical dislocation. We immediately harvested the liver and preserved a small tissue

sample in RNALater (Ambion, Inc.). The time span from the chamber opening to tissue preservation was less than 30 minutes. Within 3 hours of collecting the blood samples we centrifuged the microhematocrit tubes. Hematocrit was measured as the percentage of red blood cells in the column of blood. The two samples for each mouse were averaged before performing subsequent analyses. Variation in body mass and hematocrit were analyzed with a one-way ANOVA followed by the Ryan-Einot-Gabriel-Welsch multiple range test for pairwise tests among the means of the three groups (104). All procedures outlined above were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee (IACUC).

Liver samples were stored at  $-80^{\circ}\text{C}$  until the time of RNA purification. After homogenizing 30 mg of liver tissue using a liquid nitrogen cooled hammer and QiaShredder vials (Qiagen, Inc.), we used a Qiagen RNA Extraction Minikit (Qiagen, Inc.) to purify RNA from the tissue samples following the procedures outlined in the Qiagen RNA Extraction Minikit instruction manual. The quality and quantity of the RNA was evaluated using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA.), and all samples were found to be of acceptable quality for microarray hybridization.

#### *Microarray Experiment.*

For the purpose of minimizing individual variation, RNA samples obtained from mice were pooled. Each pooled sample contained RNA from 4 mice. With 12 mice in each treatment, this design resulted in 3 microarrays per treatment, and a total of 9 microarrays in the experiment. Because of the documented reliability of Affymetrix<sup>®</sup> products, we used the Affymetrix<sup>®</sup> GeneChip<sup>®</sup> Mouse Genome 430 2.0 Array (22, 57). The RNA sample preparations and microarray hybridization procedures were carried out by the University of Nevada Genomics

Center according to the procedures set forth by Affymetrix<sup>®</sup>. Aside from the microarray platform used, these procedures are identical to that described in Grimlet et al (38).

*GeneChip<sup>®</sup> Data Processing and Analysis.*

The Affymetrix<sup>®</sup> GeneChip<sup>®</sup> arrays were first inspected using a series of quality control steps. Images of all arrays were examined, and no obvious scratches or areas of spatial variation were observed. A visual inspection of the distributions of raw PM probe values for the nine arrays showed one outlying array (the third replicate of the 3000 m experiment). Digestion curves describing trends in RNA degradation between the 5' end and the 3' end of each probeset were examined, showing a notable outlying trend in the same outlying array, with irregular degradation between the sixth and eleventh probe pairs. The three Affymetrix quality control metrics for noise and background of this array were greater than two standard deviations away from the mean rates across all nine arrays, which led us to discard this array from further study.

For the 22,226 probesets that were detected in at least one of the eight remaining arrays, raw intensity values were processed and normalized by RMA (Robust Multi-Array Average) (48). Specifically, expression values were computed from raw *CEL* files by first applying the RMA model of probe-specific correction of PM (perfect match) probes. These corrected probe values were then normalized via quantile normalization, and a median polish was applied to compute one expression measure from all probe values. Resulting RMA expression values were  $\log_2$ -transformed. The RMA expression values and raw microarray data have been submitted to the Gene Expression Omnibus (GEO) database (series no. GSE15891).

Distributions of expression values processed via RMA of the eight arrays were similar with no apparent outlying arrays. Pearson correlation coefficients and Spearman rank coefficients were computed on the RMA expression values for each set of biological replicates, with all

coefficients ranging between 0.992 and 0.997. A principal component analysis showed a clear separation between the three experimental conditions, with more than 99% of the variability of the experiment attributed to differences between expression measures of the highest elevation and the two lower elevations (Figure 1).

To determine whether genes were expressed differentially among the three simulated altitudes, an ANOVA was performed on the RMA expression values. Data were fit to a simple linear model for a one-way ANOVA, ANOVA was performed on each probeset using the linear model, and contrasts were based on differences between the three experimental conditions (57). A multiple testing correction method (False Discovery Rate) was applied to the p-values of the F-statistic (6). Genes identified with an adjusted p-value  $p < 0.05$  were extracted for further inspection and analysis. The z-scored values (expression values normalized across all eight arrays) of these probesets were clustered using a simple hierarchical clustering procedure with the Pearson correlation coefficient as distance metric and the average agglomerative method.

The genes associated to the resulting list of 580 differentially expressed probesets were compared to previous studies on hypoxia gene expression, and a list of genes common to previous studies was compiled. Gene annotation was gathered from the Affymetrix® NetAffx Analysis Center (<http://www.affymetrix.com/analysis/index.affx>). Genes were categorized by their biological functions as classified by Gene Ontology (GO) (3), and these categories were analyzed using a series of Fisher's Exact tests to determine whether or not functional categories of the selected genes were over- or under-represented. GO functional groups were also compared with other investigations in hypoxia induced gene expression. Gene Set Enrichment Analysis (GSEA) (63) was performed as recommended by the GSEA User's Guide (<http://www.broad.mit.edu/gsea/doc/GSEAUserGuideFrame.html>) on the pathways including at least 15 probesets from the significant gene list (Supplementary Table 1). Pathways were defined

by the Broad Institute's Molecular Signatures Database C2 collection of curated gene sets from online pathway databases, PubMed, and domain experts. One thousand trials were performed, in which gene set labels were randomly permuted to estimate p-values for each pathway. The p-values were adjusted using the False Discovery Rate method (FDR), where the FDR is the estimated probability that a gene set represents a false positive finding. In the case of small sample sizes, the recommended FDR threshold is 5%. Thus, we considered only those pathways with an FDR of at most 0.05.

#### *Confirmatory Real-time qRT-PCR.*

We selected six genes showing significant differential expression between simulated altitudes to verify the microarray experiment. Beta-actin (NM\_007393) was chosen as our endogenous control gene. Previously published primer pairs for each of these seven genes were selected from Primerbank (Table 1). The efficacy of each primer pair was verified prior to performing the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) experiment. The source RNA used for amplification was derived from the same samples used for microarray hybridization. Aliquots of the same 36 RNA samples were pooled in an identical manner to that used for microarray hybridization such that we had 9 samples representing the three hypoxia treatments. These 9 pooled samples were then reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

For each gene, qRT-PCR was performed in duplicate on each cDNA sample. Genes were amplified using 1 ng of total cDNA, 150 nM of each primer (forward and reverse) and the iTaq SYBR Green Supermix with ROX reagents per manufacturer direction (Bio-Rad, Inc., Hercules, CA). PCR plates were designed such that two genes and the endogenous control gene (beta actin) along with the corresponding standard curves were run on each plate. Amplifications were

performed using an ABI 7000 PRISM Sequence Detection System (Applied Biosystems, Foster City, CA) through 40 cycles 95°C for 3 seconds and 58°C for 30 seconds. We analyzed the data using the comparative  $C_T$  method ( $\Delta\Delta C_T$  method). The  $\Delta C_T$  was calculated as  $C_{T \text{ target}} - C_{T \text{ b-actin}}$ . The  $\Delta\Delta C_T$  was  $\Delta C_{T \text{ 4500}} - \Delta C_{T \text{ 1400}}$  for each gene. The fold difference was expressed as  $2^{-\Delta\Delta C_T}$  (60).

## RESULTS

### *Physiological Responses.*

After four weeks of exposure to hypoxia, the body masses of mice differed significantly ( $p < 0.001$ ) among the three treatments. Mice from the two most hypoxic treatments (3,000 m and 4,500 m) were significantly ( $p < 0.05$ ) smaller in mass than mice from the 1,400 m treatment. This difference appeared to have occurred during the first week of exposure to hypoxia. Data for the initial starting masses are unavailable. However, because these mice are from an inbred strain, and because they were randomly assigned to treatments, it is unlikely that the starting masses were significantly different. As illustrated in Figure 2, variation in body mass was established within the first week by either a loss of body mass or a slower growth rate of hypoxic mice. The growth rates thereafter appear to be comparable among the treatments.

Whether the body mass of the 3,000 and 4,500 m mice differed depended on the inclusion of two potential outlier mice. During the course of the experiment, two of the mice that were in the 4,500 m chamber were much smaller ( $> 3.5$  g) than the other mice in that group. If those mice are excluded from the body mass analyses, then the mice in the 3,000 m and 4,500 m chambers are not significantly different from each other (mean masses of 24.6 g and 23.9 g, respectively), but both those groups of mice are significantly different in mass ( $p < 0.05$ ) from the 1,400 m group (mean mass of 26.6 g, Figure 2). If the two unusually small mice (16.9 and 19.1 g) from the 4,500 m are included in the analyses, the residuals from the analysis of variance deviate significantly from a normal distribution, which might cast doubt on that analysis. However, the nominal  $p$ -values from the analyses including all 36 mice suggest that the body mass of all three groups differed significantly ( $p < 0.05$ ) from one another. Figure 2 depicts the mean body mass over the four weeks with the two outlier mice excluded from the data set.

Hematocrit differed significantly ( $p < 0.001$ ) among the three treatments. Mice housed in the 1,400 m chamber had a mean hematocrit of 56.2 % RBC, the 3,000 m treatment had a mean hematocrit of 60.6 % RBC, and mice in the 4,500 m treatment had an incredibly high mean hematocrit of 80.7% RBC (Figure 3).

#### *Microarray Results.*

Of the 45,101 probesets on the array, 22,226 probesets were detected in at least one of the eight useable arrays. Upon the application of ANOVA and the control of the false discovery rate, 580 probesets showed significant ( $p < 0.05$  after multiple testing correction) differential expression across the three experimental conditions. Of these probesets, 455 showed an increase in expression in the 4,500 m treatment as compared to the 1,400 m treatment, with the remaining 125 having a decrease in expression in 4,500 m treatment versus the 1,400 m treatment. A heatmap representation of gene expression patterns revealed a trend of distinct differences between the high and low elevation, with approximately 85% of genes showing intermediate transcript abundances in the 3,000 m group (Figure 4). Because of this trend, we focused further investigation on the comparison between 1,400 m and 4,500 m groups.

To determine whether a specific functional group was over-represented in the set of 580 probesets that showed significant differences in expression, a series of Fisher's Exact tests was performed on twenty-one GO functional categories, each of which contained at least five probesets. After a multiple testing correction, only one category other than the "Undefined Function" category showed a significant difference (decrease) in the percentage of occurrence of functionally assigned probesets among all probesets in the analysis ( $N=14,107$ ), and those in our list of interest ( $N = 389$ ). This category, defined as "transcription", indicated that genes involved in transcription are underrepresented in hypoxic mice (4,500 m versus 1,400 m). Other than this

group, the lack of under- or over- representation of a particular functional group may be due to the small number of probesets with significant differences in expression.

Independent of the GO functional analysis above, we compiled a list of biological functional groups with more than 5 representative probesets that showed significant differential expression between the 1,400 and 4,500 m treatments. These functional groups included those associated with the immune response, angiogenesis and blood vessel development, oxygen transport, oxidation reduction (electron transport), glycolysis, protein amino acid phosphorylation, carbohydrate metabolic process, fatty acid and lipid metabolic processes, and other metabolic processes (Table 2). While the representations of these functional groups were not statistically significant in our selected subset of interest, many of these functional groups have been previously reported to be important to responses to hypoxia.

In addition to using GO term-based functional analyses, we used Gene Set Enrichment Analysis (GSEA) to investigate the over- and underrepresentation of functional groups within the genes of interest (95). GSEA is a method that tests whether gene sets, rather than individual genes or probesets, are associated with experimental conditions or classes in a given study. Definitions of gene sets are based on published and curated information on biochemical pathways. The GSEA method yielded several pathways having a statically significant (FDR value of  $< 0.05$ ) association with the up-regulation in the 4,500 m group versus the 1,400 m group. Of particular interest was the Hematopoietic Stem Cell and Adult Progenitors gene set, which is a set of genes found to be expressed during hematopoietic stem cell and hematopoietic progenitor cell development (49). The list of genes belonging to this gene set showed increased expression in the 4,500 m treatment and is presented in Table 3.

Based on previous studies of how gene expression responds to acute or cellular hypoxia, we compiled a list of genes that we suspected would be affected by chronic systemic hypoxia.

Many of these genes are regulated by the HIF pathway. However, few of these genes appeared to be significantly differentially expressed in this experiment. Indeed, in our list of 580 probesets, only 7 are known to be regulated by HIF (Table 4). When we compared our data for genes not regulated by HIF with data from a study looking at effects of acute hypoxia on hepatocytes (89), we found three genes in common with two genes showing concordant responses to hypoxia, and one gene being expressed in the opposite direction in response to hypoxia. Furthermore, only a handful of the 580 genes that were differentially expressed in our experiment have previously been reported to respond to hypoxia in any study (Table 5).

Several genes unique to hypoxia studies were expressed more than 1.5- $\log_2$  fold when comparing 4500 m and 1400 m simulated altitudes. A probeset representing leptin receptor showed the most dramatic difference in expression with a  $\log_2$  fold increase of 3.05 in 4,500 m mice as compared to 1,400 m mice. Two other probesets representing the leptin receptor also showed significant differences in expression with  $\log_2$  fold changes of 2.93 and 0.807, respectively. Consistent with the hematocrit results, a transcript representing alpha-globin showed a 1.97  $\log_2$  fold increase in expression in the 4,500 m versus the 1,400 m mice. Two other probesets representing alpha-globin and one probeset representing beta-globin also showed significant differences in gene expression, though none over the 1.5  $\log_2$  fold. Several genes encoding three families of cytochrome p450 and several genes involved in the G-protein coupled receptor protein signaling pathway were among the genes with more than 1.5  $\log_2$  fold changes (Table 6).

As mentioned earlier, relatively few genes showed decreased transcript abundance in the hypoxic conditions as compared to genes that showed increased transcript abundance. Table 7 lists 25 genes that had a decreased  $\log_2$  fold-change of more than 1.0 in the 4,500 m treatment

versus the 1,400 m treatment. These genes represent a diverse set of functions, without any readily discernable pattern.

*Confirmatory qRT-PCR.*

To validate the expression profiles obtained from the Affymetrix GeneChip™ Mouse 430 expression set array, qRT-PCR was performed on six target genes and one endogenous control gene. Each of these genes was covered by at least one probeset that appeared in our list of the 20 most differentially expressed transcripts and had known biological function. We compared transcript abundance between the 4,500 m and 1,400 m for all six genes. We performed a linear regression analysis to measure the relationship between the PCR data and the microarray data, which resulted in a very strong correlation of 0.935, and a P-value of  $p=0.006$ . This result indicates a high correlation and confirmation of our microarray results (Figure 5).

## DISCUSSION

Hypoxia is a significant physiological stressor associated with high altitude environments. Although most mammals have limited evolutionary history with hypoxic environments, mammals display various characteristic physiological responses when acclimatizing to high altitudes. This study utilized DNA microarrays (a) to help understand the gene expression changes in the liver that may underlie physiological responses following chronic exposure and (b) to identify other potential physiological effects of hypoxia that may have been overlooked previously by acute studies.

The experiment was conducted at 1400 m, not sea level. While there are some effects of this altitude on physiology (for instance, a hematocrit of 56.2 as opposed to 52 % as is average for sea level C57Bl/6 mice), this elevation is not considered to be extreme. The partial pressure of oxygen found in Reno is not expected to elicit many of the characteristic responses to high altitude that we typically see over 2000 m (103). That being said, it is important to appreciate that the variation in gene expression we report is not a comparison of hypoxia versus normoxia, but of varying degrees of hypoxia – from mild to extreme.

Based on the observed differences in body mass and hematocrit among our treatments, the hypoxic conditions were effective in eliciting significant physiological responses. However, the mRNA expression in the liver after 32 days of hypoxia was different from expression patterns reported in studies of acute hypoxia studies (37, 89, 99). While several previous studies reported similar numbers of genes with differential expression, most of the genes in this experiment exhibited increased transcript abundance, but most studies of acute or cellular hypoxia report a general trend of decreased transcript abundance (89). Thus, it appears that after more prolonged periods of hypoxia, relative steady-state transcript abundance changes from a trend of being

reduced to a trend of being increased. This result is consistent with data collected from murine hearts (25).

Few genes known to be regulated by hypoxia inducible factor are included among the 580 genes that were significantly differential expressed between our high and low altitude treatments. This result indicates that, after 32 days, the previously described stress pathways, and in particular the HIF pathway, are no longer significant factors in regulating and maintaining physiological acclimation to hypoxia. Indeed, other studies have shown that HIF-1 $\alpha$  abundance peaks after 4-5 hours in the liver, then decreases back to normal over the course of several hours (94). These findings indicate that, while HIF is an important transcription factor in regulating the initial responses to acute hypoxia, it is not as important in the maintenance of acclimatization to chronic hypoxia stress, at least in the liver. There are two non-mutually exclusive explanations for this result. One is that as animals physiologically acclimate to hypoxic environments, their tissues return to a state of oxygen homeostasis and various stress responses and the HIF pathways are no longer activated. The second is that as hypoxic exposure moves from an acute to chronic state, other genetic pathways and transcription factors are activated that regulate the specific processes associated with maintaining the acclimatized state.

Few genes that are typically reported to be responsive to hypoxia showed differential expression in our study. A possible explanation for this discrepancy is that we studied an organ *in vivo* whereas most other studies have been *in vitro*. However, the patterns of gene expression reported here are different than those reported in *in vivo* studies of murine livers exposed to either acute systemic or intermittent hypoxia (21, 56). Both of those studies of murine livers reported significant changes in lipid biosynthesis and sterol metabolism. While some genes related to these processes are differentially expressed in this study, there are not as many, nor are they the same genes. This finding indicates that chronic hypoxia induces different gene expression patterns in

liver *in vivo* than either acute or intermittent hypoxia. Indeed, recent findings confirm that intermittent and chronic hypoxia stimulate different gene expression patterns in cardiac tissue (25, 47).

The most dramatic result of this study was the large increase in hematocrit in the 4,500 m mice. While modest increases in hematocrit are generally seen as being beneficial acclimations to hypoxia (10), an increase to 80.7% RBC is extreme and much greater than expected. A hematocrit this high leads to viscous blood and can put a significant strain on the heart. Consistent with this response was the increased expression of both alpha-globin and beta-globin, the two protein subunits of hemoglobin. However, what is unclear is why these genes are expressed in the liver. There are two possible explanations for this phenomenon. One is the presence of immature erythrocytes within the liver tissue, which would express both the alpha-globin and beta-globin mRNAs. Indeed, polycythemia at the level reported here is often accompanied by increased circulation of immature erythrocytes. However, it is unclear whether there would be sufficient numbers of immature erythrocytes in the liver to elicit this response. An alternative explanation is that portions of the liver were converted to hematopoietic tissue to support the massive production of red blood cells. This hypothesis is supported by the overrepresentation of differentially expressed genes belonging to the hematopoietic stem cell gene set, as indicated by the GSEA analysis. The phenomenon of extramedullary hematopoiesis is well documented in cases of pathological anemia, such as severe thalassemia or myelofibrosis (62, 63) and it can occur when anemia is induced artificially (77). However, there is little, if any, information on this phenomenon in relation to systemic hypoxia or exposure to high altitude environments. Therefore, the incidence of extramedullary hematopoiesis and its consequences for high altitude acclimation have yet to be seriously considered. This result highlights the dynamic nature of the liver and the importance of *in vivo* studies to chronic hypoxia. The result deserves

additional study. While we were unable to conduct additional confirmatory histological studies to confirm that extramedullary hematopoiesis took place in the liver, it is one possible explanation for our results.

The transcript showing the most dramatic increase in expression in the 4,500 m treatment versus the 1,400 m treatment encoded a leptin receptor. This result is intriguing given the known responsiveness of the cytokine leptin to hypoxic stimulus. Circulating levels of leptin, a pleiotropic cytokine released by fat cells, are increased by acute hypoxia, and transcription appears to be regulated by HIF-1 (39, 61, 87, 88). However, in contrast, humans exposed to chronic high altitude hypoxia had decreased levels of circulating leptin (108). It is possible that regulation of leptin receptor is another important mechanism of regulating the leptin pathway. Indeed, leptin receptor mRNA abundance in the liver increases in response to short-term fasting and increased circulating leptin (17).

While our results indicate that the leptin pathway may be an important response to hypoxia, the pleiotropic nature of this pathway makes it difficult to interpret the physiological significance of increased leptin receptor mRNA abundance in the liver. The leptin pathway is best known for its inhibitory effect on appetite, and an obvious hypothesis would be to link the leptin pathway with hypoxia induced anorexia. However, despite increased expression during hypoxia, leptin does not appear to regulate hypoxia induced anorexia (88, 98). Leptin signaling also has a significant positive effect on organismal metabolic rate and body temperature (2, 31, 59, 69, 91). In contrast, acute hypoxia typically leads to decreased metabolic rate and body temperature, at least in small mammals (12, 29, 32, 64, 65, 90). While we did not measure metabolic rate or body temperature in our mice, it seems likely that they became hypometabolic and anapyrexia when initially exposed to hypoxia (41). However, the effects of prolonged hypoxic exposure on metabolism are less obvious as there are comparatively fewer data on the effects of chronic

hypoxia. Nonetheless, we hypothesize that at least in some strains and species, hypometabolism and anapyrexia may be transient responses to hypoxia (1, 67, 78). As an animal acclimates to hypoxia and attempts to regain oxygen homeostasis via increased oxygen delivery to its tissues, it seems plausible that metabolic rate would return toward normal. Perhaps leptin and its receptors help regulate this process? Furthermore, responses to hypoxia can be species specific (40, 42). For example, compared to effects on metabolic rates of rats, hamsters may be less affected by hypoxia and mice may be more affected (30, 41). Accordingly, hypoxia-induced changes in gene expression may also be species specific, and as for any study of a single species of mammal, our results may not be generally applicable to other mammals. Therefore we conclude that the effects of chronic hypoxia on the leptin pathway and its relationship to body temperature and metabolism is an area in need of further research.

Other physiological processes that are affected by leptin include glucose and fatty acid metabolism, hematopoiesis, and immune function (2, 31, 100). These physiological processes also appear to be important in acclimation to hypoxia. Previous studies have demonstrated that fatty acid metabolism is enhanced in rats exposed to chronic hypobaric hypoxia (68), and indeed several genes related to fatty acid metabolism were differentially expressed in our experiment. While it is unknown whether or not fatty acid metabolism was increased in our mice, it is possible that altering fatty acid metabolism is important for acclimating to hypoxia and the leptin pathway plays a role in this response. Another important function of leptin is its role in hematopoiesis. Leptin receptor can be expressed by hematopoietic stem cells, and indeed, the leptin pathway is an important progenitor of hematopoiesis (7, 26). The dramatic increased expression of leptin receptor in our 4,500 m liver samples may be another indication that extramedullary hematopoiesis is taking place.

We sorted transcripts significantly affected by hypoxia into their functional classes (Table 2). These biological functional groups include transcripts involved in angiogenesis, oxidation reduction, carbohydrate metabolism, fatty acid and lipid metabolism, glycolysis, and protein amino acid phosphorylation. Many of these functional groups typically respond to acute or intermittent hypoxia, and thus these results are not surprising (37, 56, 89). However, the particular genes representing these functional groups are not typical of previous studies of hypoxia. This result indicates that these biological functions are important in acclimation to hypoxia, but that the particular transcripts that are involved in adjusting to hypoxia vary temporally with duration of hypoxic exposure.

The metabolic activities of the liver, which often generate carbon dioxide, also play an important role in acid-base homeostasis. This process is important to hypoxia acclimatization in that the hypoxic ventilatory response typically leads to respiratory alkalosis. While increased ventilation remains during long periods of acclimatization (71), the pH of the blood typically returns close to normal via metabolic and blood regulation of bicarbonate (1, 67, 103) However, because we did not measure either ventilatory rate or blood pH, it is impossible to say whether alterations in the above transcript abundances could be contributing to maintenance of acid-base homeostasis, or be affected by the slightly higher blood pH typically found in mammals acclimatized to hypoxia. To add to the complexity, two transcripts that have been implicated in maintaining acid-base balance had decreased expression in the 4500 m mice – aquaporin 8 ( $\log_2$  fold change of -1.09) and carbonic anhydrase 14 ( $\log_2$  fold change of -0.95)(44, 50, 58). So while we are unable to draw direct conclusions regarding the relationship between liver transcript abundance and acid-base balance in response to HVR, we encourage the reader to keep this process in mind.

In this study we found an abundance of differentially expressed genes related to the immune system. Unlike the functional classes described above, the immune system is not typically viewed as important in responding to chronic, systemic hypoxia. However, there is increasing evidence that hypoxia can alter the immune system. For example, several studies of humans in high altitude environments indicate perturbations of the immune system, particularly with respect to changes in circulating leukocytes (5, 14, 15, 24). Numerous explanations have been proposed to explain why sojourners to high altitude experience immune changes, including changes in diet, stress, etc. (5). More recent evidence indicates that hypoxia in and of itself has an influence on the circulating immune system. In particular, hypoxia has a pro-inflammatory effect on macrophages, neutrophils and other leukocytes, and an anti-inflammatory effect on some lymphocytes (9, 18, 55, 66, 97, 101). Furthermore, there is growing evidence that HIF is a key regulator of many immunological processes (19, 43, 109). Yet again, the particular genes regulated by HIF pathway are not differentially expressed in our study. This result is similar to that for the other biological functional groups reported above. Namely, the immune system appears to respond to both acute and chronic hypoxia, but the particular transcripts involved in the immune systems responses vary with length of exposure to hypoxia.

The liver is a dynamic organ composed not only of hepatocytes, but also housing many immune cells, blood cells, and, perhaps in this case, hematopoietic tissue. Unfortunately, it is not possible to distinguish which responses in gene expression are attributable to which cell types or processes. This may be a major source of variation between this study and other reports of gene expression in response to hypoxia. However, this study indicates that many of the biological functional groups regulated in response to acute hypoxia are also important in maintaining acclimation to chronic systemic hypoxia. Therefore, we hypothesize that regulatory pathways, and thus the representative genes expressed from a functional group, change with time and

exposure to systemic hypoxia. So, while one set of genes achieves initial acclimatization to hypoxia, different but related groups of genes are likely involved in maintaining acclimatization. Understanding these differences will be important to better understanding the physiological effects of hypoxia and acclimatization to high altitudes.

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## TABLES

Table 1. Selected genes and primers used for qRT-PCR

<b>Ref Sequence ID</b>	<b>Common Gene Name</b>	<b>Forward Primer Sequence 5'→3'</b>	<b>Reverse Primer Sequence 5'→3'</b>
NM_007468	Apolipoprotein A-IV	CAACAGGCTGAAGGCTACGAT	CGATTTTTCGGGAGACCTTGG
NM_010704	Leptin receptor	GTCTTCGGGGTTGTGAATGTC	ACCTAAGGGTGGATCGGGTTT
NM_009063	Regulator of G Protein signaling 5	ATGGATTTGCCAGCTTCAAAAGT	GAAGTGGTCAATGTTACCTCTT
NM_008218	Hemoglobin alpha	CACCACCAAGACCTACTTTC	CAGTGGCTCAGGAGCTTGA
NM_024406	Fatty acid binding protein 4	AAGGTGAAGAGCATCATAACCCT	TCACGCCTTCATAACACATTCC
NM_007822	Cytochrome P450 4,a,14	GTCTCTCGGGGAGCAATATACG	ACCAATCCAGGGAGCAAAGAA
NM_007393	Beta actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Table 2. Genes differentially expressed by chronic hypoxia arranged in functional classes.

Functional Class	Common Gene Name	Probset ID	Log <sub>2</sub>	P-Value
			Fold Change	
<b>Immune System*</b>				
	Apolipoprotein A-IV	1417761_at	1.801	0.012
	Apolipoprotein A-IV	1436504_x_at	1.415	0.005
	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1420408_a_at	1.252	0.040
	Vascular cell adhesion molecule 1	1448162_at	1.247	0.010
	CD24a antigen	1448182_a_at	0.994	0.032
	CD38 antigen	1450136_at	0.980	0.020
	Serine (or cysteine) peptidase inhibitor, clade A, member 3G	1424923_at	0.861	0.012
	C-type lectin domain family 4, member n	1425951_a_at	0.833	0.011
	CD68 antigen	1449164_at	0.833	0.019
	Eosinophil-associated, ribonuclease A family, member 1	1422411_s_at	0.794	0.002
	Cytotoxic T lymphocyte-associated protein 2 alpha	1448471_a_at	0.779	0.011
	Defensin beta 1	1419491_at	0.751	0.019
	CD55 antigen	1460242_at	0.711	0.010
	CD163 antigen	1419144_at	0.706	0.035
	Interleukin 10 receptor, beta	1419455_at	0.704	0.004
	CD24a antigen	1416034_at	0.698	0.018
	BCL2/adenovirus E1B interacting protein 3-like	1448525_a_at	0.695	0.032
	Fc receptor, IgG, low affinity III	1448620_at	0.692	0.011
	C-type lectin domain family 4, member n	1419627_s_at	0.684	0.013
	Interleukin 13 receptor, alpha 1	1427164_at	0.662	0.015
	Collectin sub-family member 10	1457871_at	0.661	0.009
	CD48 antigen	1427301_at	0.558	0.023
	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	1460258_at	0.558	0.011
	Interleukin 6 signal transducer	1460295_s_at	0.557	0.019
	Eosinophil-associated, ribonuclease A family, member 2	1449846_at	0.551	0.035
	Attractin	1421166_at	0.517	0.034
	Serine (or cysteine) peptidase inhibitor, clade A, member 3K	1424923_at	0.501	0.035
	Interleukin 6 signal transducer	1452843_at	0.480	0.028
	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1435751_at	0.409	0.009
<b>Angiogenesis and Blood Vessel Development †</b>				
	Heme oxygenase (decycling) 1	1448239_at	1.959	0.022
	Connective tissue growth factor	1416953_at	1.385	0.013
	ELK3, member of ETS oncogene family	1448797_at	0.774	0.026
	Forkhead box O1	1416981_at	0.615	0.046
	Vascular endothelial growth factor B	1451803_a_at	0.606	0.024

Endomucin	1425582_a_at	0.600	0.020
EGF-like domain 7	1451428_x_at	0.573	0.018
Platelet derived growth factor, alpha	1418711_at	0.571	0.031
Matrix metalloproteinase 14 (membrane-inserted)	1448383_at	0.515	0.021
Endoglin	1432176_a_at	0.493	0.050
Reticulon 4	1421116_a_at	0.467	0.027
Cadherin 5	1433956_at	0.416	0.035

#### Electron Transport

Cytochrome P450, family 4, subfamily a, polypeptide 14	1423257_at	1.970	0.009
Cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	1.800	0.005
Cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	1.576	0.002
Flavin containing monooxygenase 2	1453435_a_at	1.485	0.006
Flavin containing monooxygenase 2	1435459_at	1.449	0.007
P450 (cytochrome) oxidoreductase	1416933_at	0.808	0.012
STEAP family member 4	1425829_a_at	0.672	0.039
Cytochrome P450, family 4, subfamily a, polypeptide 10	1424853_s_at	0.648	0.019
ERO1-like ( <i>S. cerevisiae</i> )	1419030_at	0.647	0.040
Cytochrome b-245, beta polypeptide	1422978_at	0.641	0.043
Glutaredoxin	1416593_at	0.617	0.009
Cytochrome P450, family 2, subfamily c, polypeptide 38	1452501_at	0.569	0.012
Cathepsin B	1417491_at	0.562	0.022
Glutaredoxin	1416592_at	0.499	0.022

#### Oxygen

##### Transport

Hemoglobin alpha, adult chain 1	1417714_x_at	1.865	0.000
Hemoglobin alpha, adult chain 1	1428361_x_at	0.641	0.019
Hemoglobin, beta adult major chain	1417184_s_at	0.593	0.015
Hemoglobin alpha, adult chain 1	1452757_s_at	0.536	0.020

#### Protein Amino Acid Phosphorylation

Wee 1 homolog ( <i>S. pombe</i> )	1416774_at	1.426	0.025
Rap guanine nucleotide exchange factor (GEF) 4	1421622_a_at	1.159	0.015
Wee 1 homolog ( <i>S. pombe</i> )	1416773_at	0.818	0.015
Endothelial-specific receptor tyrosine kinase	1418788_at	0.745	0.013
Serum/glucocorticoid regulated kinase 3	1420918_at	0.739	0.031
Protein kinase C, eta	1422079_at	0.655	0.033
Transient receptor potential cation channel, subfamily M, member 7	1416800_at	0.639	0.038
Serine/threonine kinase 3 (Ste20, yeast homolog)	1418513_at	0.523	0.034
Cyclin-dependent kinase 4	1422440_at	0.506	0.035
Tyrosine kinase receptor 1	1416238_at	0.502	0.026
Protein kinase N2	1437296_at	0.484	0.033

Receptor (TNFRSF)-interacting serine-threonine kinase 1	1419508_at	0.392	0.040
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### Metabolic Processes

#### Glycolysis

Pyruvate kinase, muscle	1417308_at	0.750	0.016
2,3-bisphosphoglycerate mutase	1448119_at	0.731	0.016
2,3-bisphosphoglycerate mutase	1415864_at	0.629	0.016
Aldolase 1, A isoform ///	1439375_x_at	0.494	0.041
Aldolase 1, A isoform	1434799_x_at	0.482	0.024
Pyruvate dehydrogenase E1 alpha 1	1449137_at	0.463	0.040

#### Metabolism

‡

Leptin receptor	1456156_at	3.051	0.002
Leptin receptor	1425644_at	2.934	0.001
CD38 antigen	1450136_at	0.980	0.002
Atpase, class VI, type 11C	1442367_at	0.935	0.006
Atpase, class VI, type 11C	1442367_at	0.935	0.006
Ectonucleotide pyrophosphatase/phosphodiesterase 1	1419276_at	0.838	0.030
Leptin receptor	1425875_a_at	0.807	0.013
RIKEN cDNA A230097K15 gene	1454799_at	0.676	0.032
Carbonyl reductase 1	1460196_at	0.656	0.009
Glutathione S-transferase, mu 3	1427473_at	0.633	0.013
Retinol dehydrogenase 9	1427963_s_at	0.624	0.022
Phosphoribosyl pyrophosphate amidotransferase	1452831_s_at	0.589	0.018
Arsenic (+3 oxidation state) methyltransferase	1431980_a_at	0.548	0.013
Dehydrogenase/reductase (SDR family) member 7	1426440_at	0.495	0.017
NAD kinase	1416249_at	0.455	0.029
RIKEN cDNA A530057A03 gene	1456208_at	0.453	0.043

#### Carbohydrate and Glucose Metabolism §

6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1416432_at	1.673	0.021
Glucokinase	1419146_a_at	0.979	0.009
Glucokinase	1419146_a_at	0.979	0.009
Glucokinase	1425303_at	0.849	0.010
Glucokinase	1425303_at	0.849	0.010
Preimplantation protein 4	1429639_at	0.784	0.046
Protein phosphatase 1, regulatory (inhibitor) subunit 2	1417341_a_at	0.624	0.024
Preimplantation protein 4	1429144_at	0.566	0.028
Pyruvate dehydrogenase kinase, isoenzyme 1	1423748_at	0.509	0.037
Membrane interacting protein of RGS16	1418444_a_at	0.489	0.035
N-acetylneuraminatase pyruvate lyase	1424265_at	0.340	0.039

#### Fatty Acid and Lipid Metabolic Process <sup>a</sup>

Cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	1.576	0.002
Lipin 1	1426516_a_at	1.280	0.009
Lipin 1	1418288_at	1.262	0.007
Very low density lipoprotein receptor	1434465_x_at	1.134	0.007
N-acylsphingosine amidohydrolase 3-like	1451355_at	0.994	0.003
ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417404_at	0.967	0.016
ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417403_at	0.844	0.024
Acyl-CoA synthetase long-chain family member 4	1427595_at	0.807	0.031
Cytochrome P450, family 4, subfamily a, polypeptide 10	1424853_s_at	0.648	0.019
Platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit	1422793_at	0.632	0.026
Acetyl-Coenzyme A carboxylase alpha	1427595_at	0.450	0.045

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\* Containing GO Biological Functional designations immune response, inflammatory response, acute phase response, defense response, chemotaxis

† Containing GO Biological Functional designations angiogenesis, blood vessel development, blood vessel maturation

‡ Containing GO Biological Functional designations metabolic process, regulation of metabolic process, one-carbon metabolic process)

§ Containing GO Biological Functional designations carbohydrate metabolism, glucose metabolic process

<sup>a</sup> Containing GO Biological Functional terms for fatty acid metabolism or lipid metabolism

Table 3. List of genes belonging to the Hematopoietic Stem Cell and Progenitors gene set that show increased expression in 4,500 m treatment.

Common Gene Name	Probeset ID	Log <sub>2</sub> Fold Change	Adj. P-Value
Ubiquitin specific peptidase 2	1417168_a_at	1.643	0.013
Regulator of G-protein signaling 3	1425701_a_at	1.509	0.019
C-type lectin domain family 14, member a	1419467_at	1.273	0.001
Coiled-coil domain containing 85B	1435589_at	1.112	0.002
Rap guanine nucleotide exchange factor (GEF) 5	1455137_at	1.110	0.009
EGF, latrophilin seven transmembrane domain containing 1	1418059_at	1.095	0.002
Peptidylprolyl isomerase C	1416498_at	0.983	0.005
ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417403_at	0.967	0.016
Ankyrin repeat domain 37	1436538_at	0.948	0.007
Rho-related BTB domain containing 1	1429206_at	0.921	0.019
Tissue factor pathway inhibitor	1452432_at	0.842	0.020
Ectonucleotide pyrophosphatase/phosphodiesterase 1	1419276_at	0.838	0.030
FYVE, RhoGEF and PH domain containing 5	1460578_at	0.760	0.040
Endothelial-specific receptor tyrosine kinase	1418788_at	0.745	0.013
Cyclin D2	1434745_at	0.693	0.010
Programmed cell death 6 interacting protein	1426184_a_at	0.692	0.021
Coiled-coil domain containing 80	1424186_at	0.674	0.009
Protein kinase C, eta	1422079_at	0.655	0.033
Gap junction membrane channel protein alpha 1	1438945_x_at	0.616	0.048
Vascular endothelial growth factor B	1451803_a_at	0.606	0.024
MAM domain containing 2	1453152_at	0.540	0.015
Thymosin, beta 10	1436902_x_at	0.521	0.022
Dynamin 1-like	1452638_s_at	0.415	0.037
N-acetylneuraminate pyruvate lyase	1424265_at	0.340	0.039
Aldehyde dehydrogenase 1 family, member L1	1424400_a_at	-0.411	0.036
ATP-binding cassette, sub-family B (MDR/TAP), member 11	1449817_at	-0.438	0.020
Endothelial cell growth factor 1 (platelet-derived)	1432181_s_at	-0.499	0.023

Table 4. Transcripts known to be regulated by HIF-1 $\alpha$  that show significant differences in abundance between 1,400 m and 4,500 m treatments after 32 days.

Common Name	Probeset ID	Log <sub>2</sub> Fold Change	P-value
6-phosphofructo-2-kinase/fructose-2,6-biphosphate 3	1416432_at	1.67	0.021
Heme oxygenase (decycling) 1	1448239_at	0.97	0.022
Lectin, galactose binding, soluble 3	1426808_at	0.83	0.015
Pyruvate kinase, muscle	1417308_at	0.75	0.016
Insulin-like growth factor binding protein 3	1458268_s_at	0.54	0.029
Aldolase 1, A isoform	1434799_x_at	0.48	0.024
Presenilin 2	1425869_a_at	-0.68	0.009

Table 5. Transcripts showing differential expression in response to chronic hypoxia that have previously been reported to respond to hypoxic stimulus.

Common Gene Name	Probeset ID	Log <sub>2</sub> Fold Change	P-value	References
Cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	1.800	0.005	(56)
CD24a antigen	1416034_at	0.994	0.011	(83)
Heat shock protein 110	1423566_a_at	0.772	0.009	(89)
CD24a antigen	1448182_a_at	0.70	0.019	(82)
Superoxide dismutase 3, extracellular	1417633_at	0.657	0.012	(96)
ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 1 polypeptide	1439036_a_at	0.585	0.033	(53)
Caveolin, caveolae protein 1	1449145_a_at	0.541	0.036	(82)
Pyruvate dehydrogenase kinase, isoenzyme 1	1423748_at	0.509	0.037	(89)
Caspase 1	1449265_at	0.450	0.027	(110)
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1425631_at	-0.937	0.044	(89)
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1433691_at	-1.096	0.013	(89)

Table 6. List of genes showing greater than 1.5 log<sub>2</sub> fold increased transcript abundance in response to chronic hypoxia.

Common Name	Probeset ID	Log <sub>2</sub> Fold Change	Adjusted P-value
Leptin receptor	1456156_at	3.05	0.002
Leptin receptor	1425644_at	2.93	0.001
Regulator of G-protein signaling 16	1426037_a_at	2.26	0.024
Regulator of G-protein signaling 5	1420941_at	2.24	0.000
Fatty acid binding protein 4, adipocyte	1417023_a_at	2.21	0.000
G protein-coupled receptor 98	1425314_at	2.19	0.000
Regulator of G-protein signaling 16	1451452_a_at	2.07	0.027
---	1417466_at	2.01	0.000
Cytochrome P450, family 4, subfamily a, polypeptide 14	1423257_at	1.97	0.009
Hemoglobin alpha, adult chain 1	1417714_x_at	1.87	0.000
Fatty acid binding protein 4, adipocyte	1451263_a_at	1.86	0.004
Synuclein, alpha	1436853_a_at	1.86	0.000
Predicted gene, ENSMUSG00000073738	1439816_at	1.84	0.000
Regulator of G-protein signaling 5	1420940_x_at	1.84	0.000
Apolipoprotein A-IV	1417761_at	1.80	0.005
Cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	1.80	0.005
ATP-binding cassette, sub-family D (ALD), member 2	1438431_at	1.76	0.005
Major facilitator superfamily domain containing 2	1428223_at	1.73	0.019
ATP-binding cassette, sub-family D (ALD), member 2	1419748_at	1.71	0.008
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1416432_at	1.67	0.021
Ubiquitin specific peptidase 2	1417168_a_at	1.64	0.013
Von Willebrand factor homolog	1435386_at	1.63	0.004
Synuclein, alpha	1418493_a_at	1.58	0.010
Cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	1.58	0.002
---	1417466_at	1.57	0.000
Regulator of G-protein signaling 3	1425701_a_at	1.51	0.019

Table 7. List of genes showing more than 1.0 log<sub>2</sub> fold decreased in transcript abundance in response to chronic hypoxia.

Common Gene Name	Probeset ID	Log <sub>2</sub> fold change	P-value
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427351_s_at	-1.60	0.028
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427329_a_at	-1.58	0.030
Tubulin, beta 2a	1427347_s_at	-1.57	0.009
Macrophage activation 2 like	1438676_at	-1.46	0.005
Macrophage activation 2 like	1447927_at	-1.44	0.003
Oligodendrocyte transcription factor 1	1416149_at	-1.28	0.007
Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	1420722_at	-1.27	0.002
UDP glucuronosyltransferase 2 family, polypeptide B38	1423397_at	-1.25	0.011
Early growth response 1	1417065_at	-1.22	0.011
RIKEN cDNA 2310043N10 gene	1428083_at	-1.11	0.031
RIKEN cDNA 1810046K07 gene	1453547_at	-1.10	0.003
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	1433691_at	-1.10	0.013
Aquaporin 8	1417828_at	-1.10	0.001
cDNA sequence BC015286	1457619_at	-1.04	0.011
Carboxylesterase 2	1424245_at	-1.02	0.001

**FIGURE LEGEND**

Figure 1: Principal component analysis of the eight Affymetrix arrays.

Note that 99.5% of the experimental variation is associated with the first principal component, and it indicates the separation between the highest altitude group and the other two groups.

Figure 2: Mean body mass of mice over 30 days of residence in hypoxia chambers simulating three altitudes. By day 9, 4500 m mice were significantly smaller ( $20.38 \pm 1.20$ ), than 3000 m mice ( $22.37 \pm 1.35$ ), and both 4500 m and 3000 m mice were significantly smaller than 1400 m mice ( $24.24 \pm 1.35$ ) ( $P < 0.001$ ). This pattern remained until the end of the experiment, but the differences between the 3000 m and 4500 m experiments were no longer significant. After 30 days, mice in the 4,500 m ( $23.9, \pm 0.91$ ) and 3,000 m ( $24.6 \pm 1.40$ ) chambers were significantly smaller than mice in the 1,400 m chamber ( $26.6 \pm 1.34$ ) ( $P < 0.001$ ).

Figure 3: Mean hematocrits of mice after 32 days in hypoxia chambers. All hematocrits are significantly different ( $P < .001$ ) with the 4500 m mice having a mean hematocrit of 80.9% RBC ( $\pm 3.4\%$ ), the 3,000 m group having a mean hematocrit of 60.6% RBC ( $\pm 1.9\%$ ) and the 1,400 m group having a HCT of 56.1% ( $\pm 1.4\%$ ).

Figure 4: Heatmap of 580 differentially expressed probesets. Values are normalized across the eight arrays, and clustered via a simple hierarchical clustering procedure.

Figure 5: Confirmatory real-time qRT-PCR of 6 Transcripts. Comparison between the expression values from the Affymetrix GeneChip Mouse Expression Set 430 and expression values from quantitative real time PCR. LepR is leptin receptor; FABP is fatty acid binding protein; RGP5 is regulator of G protein signaling 5; P450 is Cytochrome P450 family 4, subfamily a, protein 14; HbbA is hemoglobin alpha; APO is apolipoprotein.

## FIGURES

Figure 1.



Figure 2.

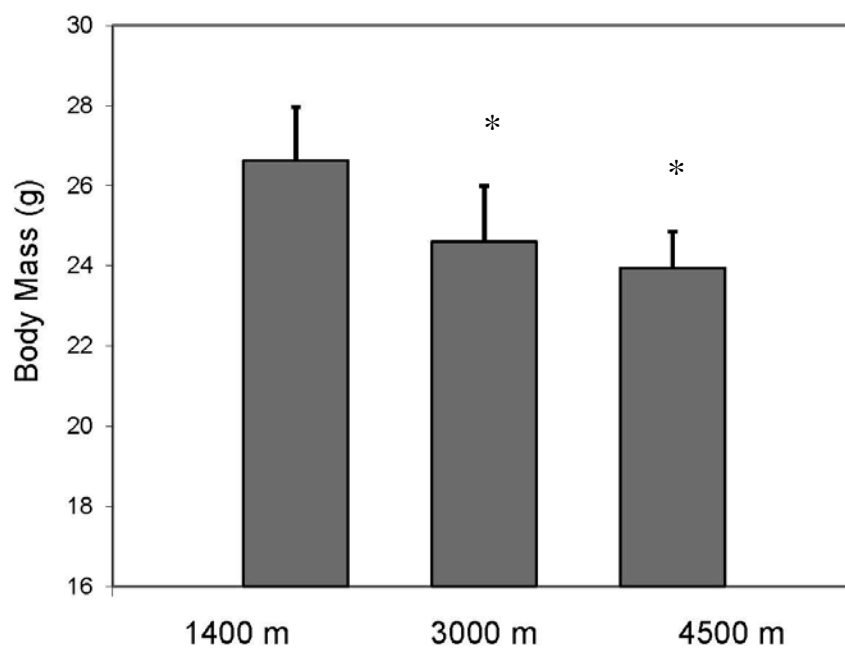


Figure 3.

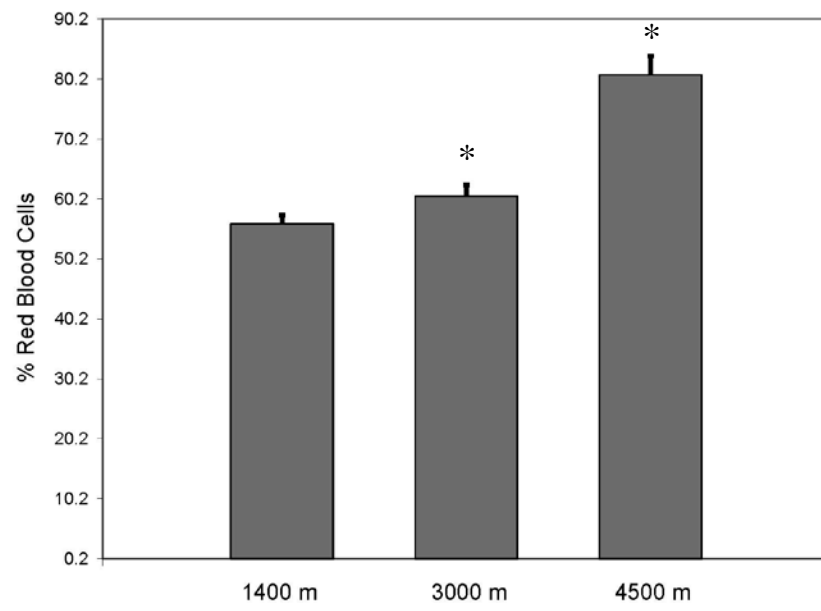


Figure 4.

Heatmap of Clustered 580 Differentially Expressed Probesets

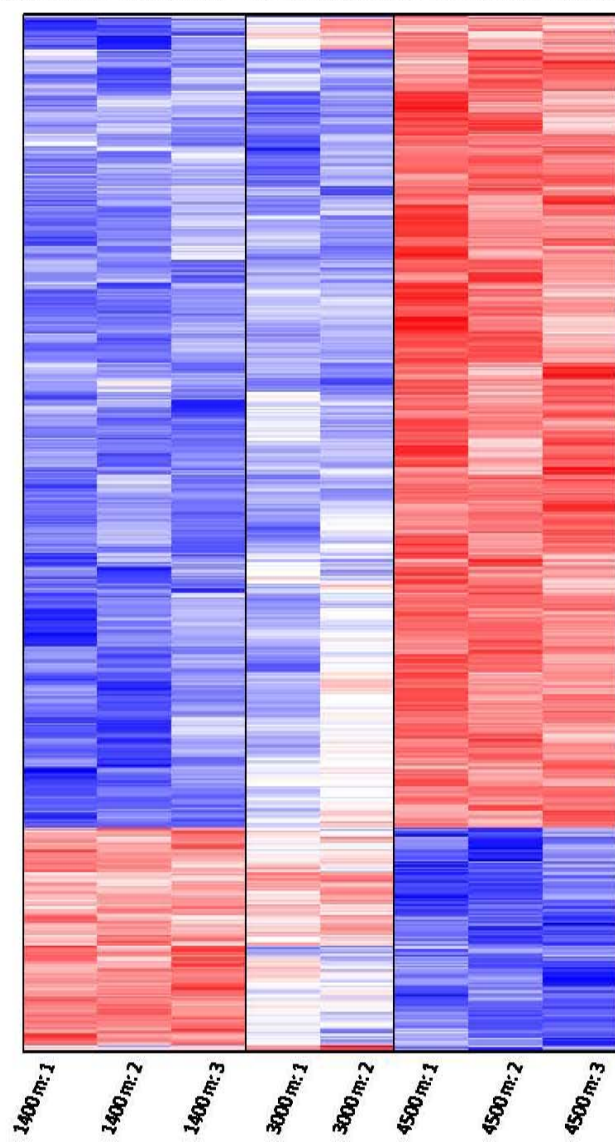
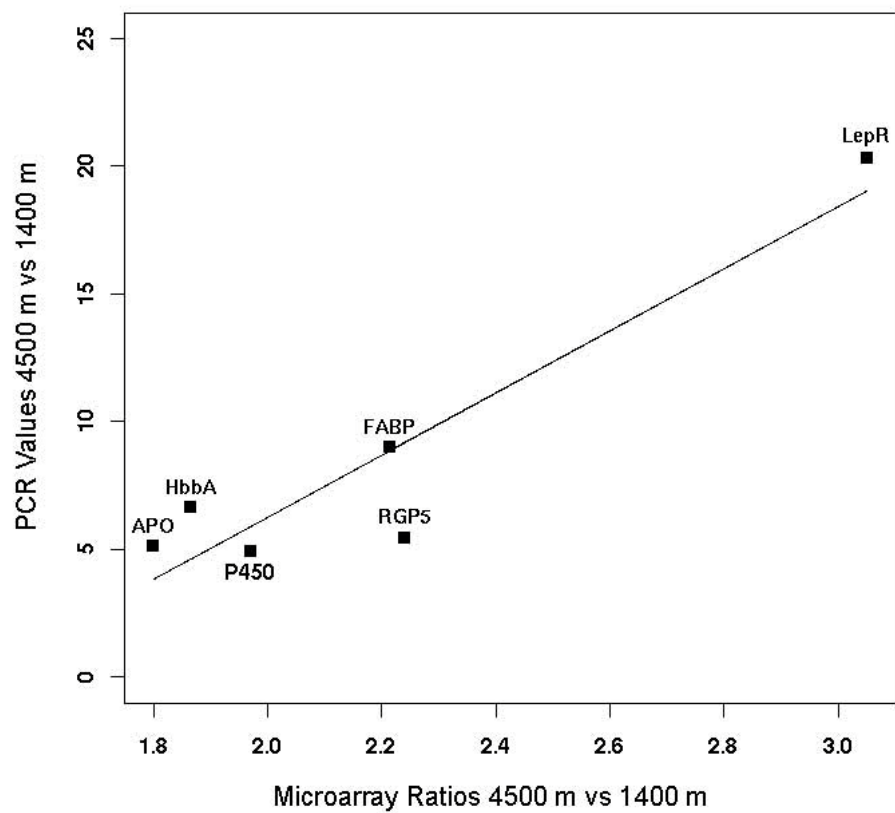


Figure 5.



**CHAPTER 2: VARIATION IN GENE EXPRESSION BETWEEN WILD HOUSE MICE (*MUS MUSCULUS DOMESTICUS*) AT HIGH AND LOW ALTITUDES.**

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**ABSTRACT**

Analysis of gene expression profiles is an attractive method for discovering how animals respond to environmental challenges in nature. Compared to low altitudes, high altitudes are characterized by reduced partial pressures of oxygen (hypoxia) and cooler ambient temperatures. To better understand how mammals cope with high altitudes, we trapped wild house mice (*Mus musculus domesticus*) from 3 populations in La Paz, Bolivia (3000 - 3600 m) and 3 populations in Lima, Peru (0 – 200 m). Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays were used to measure mRNA abundance in the livers of these mice. Only 121 probesets differed with statistical significance in their expression between low and high altitude. This number of probesets is surprisingly low given the substantial environmental differences between the two altitudes. Few of the genes that were differentially expressed have previously been associated with hypoxic or high altitude responses. However, consistent with recent evidence of adaptation in the eglin nine homolog 1 gene (EGLN1) and its expression in humans at high altitudes, expression of the gene EGLN3 was decreased in mice living in La Paz, Bolivia. This result indicates that similar evolutionary patterns may be occurring in mice as in humans, and that house mice may be useful model organisms for high altitude research. Compared to low altitude mice, high altitude mice also had increased expression of genes involved in the immune system and decreased expression of genes associated with growth, development and cell proliferation. Although the particular genes were not the same, the pattern of increased expression of immunological genes is consistent with previous microarray studies on laboratory mice acclimated to hypoxia. The immune system is not often considered an important aspect of physiological acclimation or adaptation to hypoxia, yet this study adds to increasing evidence that the immune system can play an important role in coping with high altitude life.

## INTRODUCTION

When faced with environmental change, animals cope by adjusting their physiology and behavior (i.e., by acclimatizing or acclimating to new environmental conditions). Underlying these adjustments of physiology and behavior are changes in gene expression (Gracey, 2007). There appears to be considerable genetic variation in gene expression, hence longer-term Darwinian adaptation to new environments is likely another mechanism (besides acclimatization) by which animals cope with environmental change (Whitehead and Crawford, 2006b). Therefore, it is not surprising that physiological ecologists are studying gene expression to better understand how organisms respond to physiological stress over ontogenetic and evolutionary time (Gibson, 2008; Gracey and Cossins, 2003; Roelofs et al., 2008; Shiu and Borevitz, 2008; Whitehead and Crawford, 2006a) .

How mammals cope physiologically with the multiple stresses of high altitude environments has been of great interest to ecologists and physiologists alike (Beall, 2000; Hayes and O'Connor, 1999; Hochachka et al., 1991; Lenfant, 1973; Levine and StrayGundersen, 1997; Monge and Leonvelarde, 1991; Rezende et al., 2005). At higher altitudes mammals must often cope simultaneously with colder temperatures, increased solar radiation, and decreased partial pressures of oxygen (hypoxia). While decreased temperature is not a novel physiological stress, maintaining body temperature at high altitudes can be metabolically challenging when oxygen availability is low. Furthermore, while mammals can behaviorally escape or mediate the severity of the thermal stress they experience, they are incapable of escaping hypoxic stress— mammals must meet the challenge of hypoxic stress physiologically. Therefore, high altitudes are chronic physiological stressors for the mammals that live there. A further challenge for mammals at high altitude is that many species have limited or no evolutionary history with hypoxic environments. Aside from burrowing mammals, most mammals rarely experience chronic, systemic hypoxia.

Yet despite this limited exposure, when faced with hypoxia, mammals share a suite of physiological responses that help meet the challenge of maintaining oxygen homeostasis. These classic responses include increased hematocrit, increased ventilation rate, (hypoxic ventilatory response, or HVR), increased angiogenesis, and a variety of other mechanisms that act to increase oxygen delivery to the tissues (Sarkar et al., 2003; Ward et al., 2000). In addition, some mammals, which have permanently colonized high altitudes have apparently adapted evolutionarily to hypoxia. For example, deer mice, chinchillas and guinea pigs from high altitudes have hemoglobin with increased oxygen binding affinity as compared to low altitude populations (Ostojic et al., 2002; Snyder et al., 1988). In deer mice, the exact genetic mutations in alpha-globin have been pinpointed (Storz et al., 2007). For other mammals, while some likely adaptations to high altitude hypoxia have been identified, pinpointing the genetic source or genomic underpinnings of potential adaptations has proven to be difficult (Beall, 2007; Strohl, 2008). Hence, our understanding of how mammals physiologically adjust to and evolutionarily adapt to high altitudes is incomplete.

One way our understanding of physiological responses to high altitude can be improved is by identifying how altitude affects gene expression (or the transcriptome). Whole genome gene expression profiling can be performed in a straightforward, high-throughput manner by using DNA microarrays. DNA microarrays can identify genes that are differentially expressed across altitudes, allowing us to pinpoint putative physiological processes involved in high altitude. Thereby, we can focus our efforts on candidate genes that may underlie genotypic adaptation as well as acclimatization to high altitude (Powell, 2003).

While DNA microarrays are powerful tools for studying gene expression, they are not always readily available for non-model organisms. Researchers have successfully circumvented this problem in two ways: they have created novel microarrays for the species of interest, or used

commercially available microarrays for model organisms on closely related non-model species (Cheviron et al., 2008; Gracey et al., 2001; Oleksiak et al., 2002). Unfortunately, both of these approaches have complications and limitations (Buckley, 2007; Oleksiak et al., 2001). Another approach for researching ecologically based questions is to study wild, natural populations of model organisms. Accordingly, we studied wild or feral house mouse (*Mus musculus* spp.), a species found at a wide range of altitudes in nature and also a species for which microarrays were already available.

While the commercial availability of genomic tools for house mice is one advantage, another advantage of studying house mice is that they have a suite of characteristics that make them well suited for ecological and evolutionary studies (Berry and Scriven, 2005; Galtier et al., 2004). Their evolutionary history and biogeography are closely tied with human agriculture and are well documented (Berry, 1987; Boursot et al., 1993; Silver, 1995). Currently, *Mus musculus* spp. has a worldwide commensal distribution, and mice can live in high density in urban and agricultural settings. The history of the house mouse in South America makes them particularly useful for understanding how colonizing mammals acclimatize and adapt to the stresses of new environments. House mice arrived in the South American continent with European settlers and agriculture, placing a temporal limit on their occupation to 500 years or roughly 1000 generations. House mice have been reported to live in South American mines higher than 4000 m (Hopkins and Powell, 2001) and are abundant in high altitude cities. Herein, we utilized natural populations of house mice in La Paz, Bolivia (3000 -3600 m) and Lima, Peru (sea level) to study variation in gene expression between high altitude and low altitude populations, and to identify gene products and physiological processes that might be important for high altitude life.

## METHODS

Mice were trapped in three locations each in La Paz, Bolivia (3000 - 4000 m) and Lima, Peru (0 – 200 m) in April and May of 2004. La Paz has a population of over 1 million people, and is characterized by mild temperatures. Average yearly temperatures are 5 °C to 10 °C at night and 20°C to 23°C during the day. Lima has a population of over 7.6 million people and is slightly warmer than La Paz. Average yearly temperatures are 15 °C to 20 °C at night and 18 °C to 27 °C during the day. In both cities, mice were trapped at zoos, in livestock yards, and in museum gardens and buildings. Trap sites ranged from 3000 m to 3600 m in La Paz and from 0 to 200 m in Lima. Sherman live traps were set in these locations in the evenings and then checked the following morning. Mice identified as *Mus* were transferred to the laboratory where they were weighed and examined for sex and age class. Blood was sampled from the infraorbital sinus and then mice were sacrificed via cervical dislocation. The livers were immediately extracted, placed in RNA Later (Ambion, Inc.) and stored at -20 °C until shipment to back to the University of Nevada, Reno. Within two hours of collection, blood samples were centrifuged and hematocrits were measured in duplicate for each mouse. Genetic analysis of the control and cytochrome *b* regions of mtDNA revealed all mice belonged to the *M. m. domesticus* subspecies, and that their population genetic diversity is similar to that of populations of house mice in Europe (Storz et al., 2007)

### *Sample preparation and microarray hybridization.*

Once back at the University of Nevada Reno, liver samples from 9 adult male mice from each city (total N = 18 mice) were selected for microarray analysis. From each city, three mice came from the zoos, three mice came from the livestock yards, and three mice came from museum gardens. This sampling strategy reflects a nested experimental design, where each altitude is represented by three populations and each population is represent by 3 individual mice.

Total RNA was extracted from 30 mg of liver from each mouse. Liver samples were homogenized using a liquid nitrogen cooled hammer and QiaShredder vials (Qiagen, Inc.). Qiagen RNA Extraction Minikit (Qiagen, Inc.) was then used to purify total RNA from the homogenized tissues following manufacturer protocols. The resulting total RNA samples were shipped to the Stowers Institute (Kansas City, MO) where the concentration and quality of RNA were determined by measuring absorbance at 260, 280, and 230 nm on an Ultraspec 3100 pro spectrophotometer (GE Healthcare/Amersham Biosciences, Piscataway, NJ). The purity and integrity of the RNA samples were further validated with the RNA 6000 Nano Assay and RNA LabChips on a 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA.). Microarray hybridization was carried out on the Affymetrix GeneChip® Mouse Genome 430 2.0 Array following the protocols set forth by Affymetrix. Specifically, RNA samples were prepped using 5 µg of total RNA and the GeneChip® One-Cycle Target Labeling and Control Reagents (Affymetrix, P/N 900493). A of 300 µl hybridization cocktail was prepared, including 20 µg fragmented/biotin-labeled cRNA and probe array controls. GeneChip® Mouse Genome 430 2.0 arrays were hybridized with 200 µl hybridization cocktail for 16 hours. Arrays were stained and washed using the GeneChip Fluidics Station 450 and scanned with the GeneChip Scanner 3000. Image data on each array was scaled to 150 target intensity using GeneChip Operating Software (GCOS).

*GeneChip® Data Processing and Analysis.*

The Affymetrix GeneChip® arrays were processed as described in Baze et al 2010. Specifically, expression data underwent a series of rigorous quality control steps to ensure data reproducibility and overall quality. Average background and noise metrics were examined for consistency across all 18 arrays as indicated by the Affymetrix GeneChip® Operating Software

Users Guide. All arrays were inspected using a series of quality control steps. Images of all arrays were examined, and no scratches or areas of spatial variation were observed. Digestion curves describing trends in RNA degradation between the 5' end and the 3' end of each probeset were examined, showing uniform trends in all arrays. GeneChips were then processed by RMA (Robust Multi-Array Average; (Irizarry et al., 2003)) using the R package affy (Gautier et al., 2004). The RMA expression values and raw microarray data have been submitted to the Gene Expression Omnibus (GEO) database (series no. GSE29097). Distributions of expression values processed via RMA of all arrays were similar with no apparent outlying arrays. Pearson correlation coefficients across sets of biological replicates ranged between 0.976 and 0.987. A principal components analysis (PCA) unfortunately did not show clear separation between the two altitudes nor populations (Figure 1).

We performed two different ANOVA analyses to test whether expression measures differed between low and high altitude. Our first approach was to treat individual mice as subsamples of each population (i.e., where the three mice studied represented subsamples of the population from the location in which they were trapped). This approach treated the experimental design as a nested ANOVA where individual mice are nested within their respective populations and where the main effect or treatment is altitude. Our second approach was to ignore possible effects of populations/locations and to use a one-way ANOVA to compare expression in low and high altitude mice. A multiple testing correction method (False Discovery Rate) was applied to the p-values of both statistical tests (Benjamini and Hochberg, 1995). Genes identified with an adjusted p-value  $p < 0.05$  were extracted for further inspection and analysis.

Gene annotation was gathered from the Affymetrix NetAffx Analysis Center (<http://www.affymetrix.com/analysis/index.affx>). The probesets identified as being differentially expressed were compared to lists of genes previously reported to be involved in hypoxic

responses. Genes were then categorized by their biological functions as classified by Gene Ontology (GO). We used the online web tool GoMiner to conduct a series of Fisher's Exact tests designed to determine whether or not functional categories of the selected genes were over- or under-represented (Zeeberg et al., 2003; Zeeberg et al., 2005). Independent of GoMiner, probesets were sorted by their GO functional groups, and groups containing several probesets were considered for interpretation.

*Confirmatory qRT-PCR.*

To verify the microarray experiment, five genes whose expression differed significantly between low and high altitude were analyzed with quantitative real time PCR. Because it is not known to be responsive to hypoxia, beta-actin (NM\_007393) was chosen as our endogenous control gene. Primer pairs for each of these genes were selected from Primerbank (Spandidos et al., 2010; Wang and Seed, 2003) (Table 1). The efficacy of each primer pair was verified prior to performing the qRT-PCR experiment at AGCT, Inc. (Wheeling, IL.)

The source RNA used for amplification was derived from the same samples used for microarray hybridization. Three samples (one sample from low altitude, and two from high altitude) did not have sufficient quantities for qRT-PCR, and therefore were not used in the analysis. From the remaining 15 mRNA samples were sent to AGCT, Inc. (Wheeling, IL) for the qRT-PCR analysis. From each of the RNA samples, cDNA was synthesized from 500 – 800 ng of total RNA with oligo dT primer and SuperScript Double Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). qRT-PCR was performed in triplicate for each of the 7 genes on each cDNA sample. Genes were amplified and  $C_T$  scores were generated using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Using the  $C_T$  scores generated by AGCT, Inc, expression values were determined using the comparative  $C_T$  method ( $\Delta\Delta C_T$

method). The  $\Delta C_T$  was calculated as  $C_{T \text{ target}} - C_{T \text{ b-actin}}$ , and  $\Delta\Delta C_T$  was calculated as  $\Delta C_{T \text{ high altitude}} - \Delta C_{T \text{ low altitude}}$  for each gene. Fold difference in gene expression were expressed as  $2^{-\Delta\Delta C_T}$  (Luu-The et al., 2005).

## RESULTS

### *Physiological Responses.*

When analyzed with a nested ANOVA, the body masses of high altitude mice were significantly greater than the masses of low altitude mice ( $P < 0.032$ ). Overall, high altitude mice had a mean body mass of 17.8 g, and low altitude mice had a mean body mass of 14.2 g (Figure 2). The hematocrits of the high altitude mice were also significantly higher than the mice trapped at low altitude. High altitude mice had a mean hematocrit of 56.5% RBC, whereas low altitude mice had a mean hematocrit of 49.3% RBC ( $P < 0.004$ , nested ANOVA) (Figure 3).

### *Microarray Results.*

Of the 45,101 probesets on the array, 26,293 probesets were detected in at least one of the 18 arrays. A nested ANOVA with control for false discovery rates indicated that no genes were significantly differentially expressed between the altitudes. Because of the large variability apparent among individuals, among population and between altitudes (Figure 1), our sample sizes were likely insufficient to detect differences in altitude given our experimental design. Therefore, we considered results from the nested ANOVA without applying the false discovery rate correction. Instead, a more stringent cutoff value of the unadjusted p-value ( $p < 0.01$ ) was used to identify genes of potential biological interest. This approach yielded a list of 81 probesets, 39 of which showed decreased transcript abundance and 42 showing increased expression in high altitude mice.

Analysis of expression data using a simple one-way ANOVA with control of the false discovery rate yielded a list of 110 probesets. While this approach yielded more probesets showing significant differential expression, it also assumes that individual mice are independent samples and ignores the effect of source population might have on gene expression. Therefore we opted to combine the probesets generated from both the nested ANOVA approach using a

cutoff of an unadjusted  $p < 0.01$ , and the one-way ANOVA approach with a cutoff of an FDR adjusted  $p$ -value of  $p < 0.05$ . This procedure led to a list of 121 probesets. Of these probesets, 76 were shared between the nested ANOVA ( $p < 0.01$ ) and the one-way ANOVA ( $p < 0.05$ ) results, 32 probesets were unique to the one-way ANOVA, and 13 probesets were unique to the nested ANOVA (Supplemental Table 1). Roughly half of these these 121 probesets showed increased expression at high altitude (62 probesets), and the other half showed decreased expression (59 probesets). This combined list was used for further functional analyses and interpretations.

To test for an over- and under-representation of GO Biological functional groups, we used the web based program GoMiner to perform Fisher's Exact tests on the expressed functional groups. After a false discover rate multiple test correction, no functional groups appeared to be over-represented in the combined gene list. This is likely due to low number of genes with significant differential expression in our list.

While there are no biological functional groups based on GO terms that appear to have statistically significant representation, there are some groups that are represented by several probesets. While not statistically significant, these groups might have biologically significant meaning for living at high altitudes. These groups include various metabolic processes; protein production, regulation and destruction; cellular processes and signaling; DNA maintenance, replication and repair; reproduction and development; and immune system processes (Table 3). The most striking of these groups are genes involved in reproduction and developmental and those belonging to the immune system. Of the 6 probesets involved in reproduction and developments, all but 1 showed decreased expression at high altitude. Of the 16 probesets belonging to immune system processes, all but 1 showed increased expression at high altitude (Table 3). Strongly represented in the list of immune response genes are probesets representing different immunoglobulin chains, seven of which show greater than two-fold expression at high

altitude. These include the kappa light chain of immunoglobulin, two heavy chains for two immunoglobulin isotypes— those for immunoglobulin M (IgM) and immunoglobulin A (IgA) - and the immunoglobulin joining chain (IgJ). IgM is the first antibody isotype produced by B cells, and circulates in a secreted form as a pentameric molecule joined by IgJ. IgA is the third isotype produced by B-cells, and forms a dimeric molecule in with the help of IgJ (Koshland, 1985). All immunoglobulin chains necessary for constructing polymeric IgM and IgA antibodies were over expressed at high altitude.

Twenty five probesets showed transcript abundance that differed more than two-fold between high and low altitudes, with 11 of these having increased expression at high altitude, and 14 having increased expression at high altitude. Interestingly, 7 these genes showing greater than 2 fold expression are involved in the immune response, specifically the adaptive immune response. Of the 11 genes showing expression levels that were decreased more than 2 fold at high altitude, 2 belonged to the reproduction and development group. However, the differences were only significant with the one-way ANOVA.

Six genes related to the 121 probesets in our list have previously been reported as hypoxia responsive genes. Three probesets had been identified in a previous microarray study of inbred mice acclimating to chronic systemic hypoxia (Baze et al., 2010). Two of these genes showed decreased expression in the mice acclimated to chronic systemic hypoxia, but showed increased expression in this experiment (Table 2). Of the genes known to be regulated by HIF, none were represented in our list of 121 genes. However one gene, EGLN3, is a known upstream regulator of HIF.

#### *Confirmatory qRT-PCR.*

Quantitative real-time RT-PCR was performed on five target genes and one endogenous control gene ( $\beta$ -actin) to validate the the expression values from the Affymetrix GeneChip<sup>®</sup>

Mouse 430 expression set array. Due to insufficient RNA quantities, two samples from high altitude and one sample from low altitude were not included in the analyses. Transcript abundance for the remaining 7 mice derived from the high altitudes and 8 mice derived from low altitudes were compared for all 5 genes. A linear regression analysis to measure the relationship between the PCR data and the microarray data resulted in a correlation of 0.82 at  $p < 0.088$ . This result indicates a sufficient correlation and confirmation of our microarray results (Figure 4).

## DISCUSSION

Mammals living at high altitudes are likely to differ from their low altitude counterparts with respect to their ability to cope with the stresses of cold, solar radiation, and hypoxia. Differences in gene expression might underlie the differences in these physiologies. This study sought to characterize the gene expression of mice living at high altitude as compared to low altitude to help identify 1) genes that might be important in colonizing high altitudes, and 2) physiological processes that have not been identified previously as important to living at high altitudes.

At tropical locations, such as La Paz, Bolivia versus Lima Peru, the differences in temperature between sea level and 3600 m are less severe than at temperate latitudes. Furthermore, while La Paz is slightly cooler than Lima, La Paz also experiences seasonal differences in temperature whereas Lima is relatively stable throughout the year. We will consider differences in transcriptomic profiles in terms of coping with hypoxia, cooler temperatures, and seasonal changes in climate.

### *Physiological Differences*

House mice at high altitude were physiologically different from house mice at low altitude. For instance, mice living at high altitude had significantly higher hematocrits than mice living at low altitude. This is a classic sign of exposure to hypoxia, and it indicates that hypoxia had a significant effect on the physiology of high altitude house mice. The body masses of the mice at high altitude were also greater than those of mice at low altitude. This result was somewhat surprising given that most small mammals show a decrease in body mass when exposed to hypoxia (Bozzini et al., 2005; Hammond et al., 2002; Quintero et al., 2010). However, there is less information regarding how body mass is affected by lifetime exposure to hypoxia, or

trends in evolutionary adaptation of body mass to high altitude. Indeed, larger body masses might be more advantageous at high altitudes due to the reduced mass specific metabolic rate (Everett and Crawford, 2010; Singer, 2004). In addition, the cooler climate of La Paz might also contribute to the larger body sizes of the mice. Indeed, Bergmann's Rule postulates that animals living in colder climates are often larger than the counterparts living in warmer climates (Bergmann, 1847; Meiri and Dayan, 2003). Because the temperature differences between La Paz and Lima are relatively small, at least compared to the temperature differences that would be expected over a similar altitudinal range in temperate regions, it is unlikely that the cooler climate in La Paz is the only factor accounting for the larger body mass of mice at high altitude. Why body mass was larger at high altitude is unclear, but it might be the result of multiple environmental and ecological factors. Alternatively, it is possible that mice at high altitude were on average older, and hence larger, than mice at low altitude. While similar numbers of juvenile mice were captured at both altitudes, low altitude juveniles were on average smaller than high altitude juveniles.

#### *Differences in Gene Expression.*

Like other microarray studies conducted on natural populations of animals, individual variation in gene expression of our mice was quite high (Crawford and Oleksiak, 2007; Oleksiak et al., 2002; Whitehead and Crawford, 2005). In this study, among population variation was also high (Figure 3). However, by combining the results from a nested and one-way analysis approach, we were able to generate a list of 121 probesets representing 107 unique genes.

Perhaps the most interesting difference in gene expression between altitudes was the 2.10 fold decreased expression of EGLN homolog 3 (EGLN3). EGLN3 belongs to the EGLN family of HIF proyl hydroxylase proteins (EGLN1, ELGN2, EGLN3, also known as PHD1, PHD2, and PHD3) (for review, Freeman et al., 2003). These enzymes are involved in the

posttranscriptional negative regulation of hypoxia inducible factor (HIF) in the presence of oxygen (Epstein et al., 2001). While the three EGLN isoforms have overlapping function, they vary in tissue specific expression patterns (Lieb et al., 2002). More specifically, knock-down of ELGN1 and ELGN3 in the liver leads to increased HIF activity, thereby leading to increased erythropoiesis (Fisher et al., 2009; Takeda et al., 2008). Hypoxia induces the expression of EGLN1 and more dramatically EGLN3. This induction of a negative regulator of HIF by hypoxia is thought to set stage for immediate degradation of HIF once oxygen levels are restored (Cioffi et al., 2003; Epstein et al., 2001).

That EGLN3 showed decreased expression levels in a hypoxic environment is counter to previous studies, yet implies potentially important regulatory changes to HIF. The decreased expression of EGLN3 could lead potentially to higher levels of HIF activation, thereby increasing erythropoiesis and other physiological responses to hypoxia. Such a response in ELGN3 expression might be of benefit to mammals permanently residing in hypoxic environments. This idea is supported by two independent findings of allelic variations in the ELGN1 isoform associated with humans adapted to high altitudes in the Himalayas (Aggarwal et al., 2010; Simonson et al., 2010). These ELGN1 alleles are found at high frequency at high altitude, and they are associated with decreased hematocrit and decreased susceptibility to high altitude pulmonary edema (HAPE). Furthermore, alternative alleles found at lower altitudes are associated with increased expression of ELGN1 at high altitude and with increased incidence of HAPE. These findings are consistent with the decreased expression of EGLN3 expression in our high altitude mice, and they suggest that EGLN3 might also play a role in responding to high altitude hypoxia.

While decreased EGLN3 expression might lead to more sensitive activation of HIF, there is little evidence that HIF is acting on its target genes. Of the rest of the genes in our list showing

differential expression to hypoxia, none are known to be HIF inducible, and few are associated with responses to hypoxia or have been reported in other hypoxia related gene expression studies. A comparison with our laboratory study on gene expression after acclimation to chronic hypoxic in C57BL/6 revealed four genes in common between the two experiments, however, three were expressed in the opposite direction (Baze et al., 2010) (Table 1.).

With the decreased expression of EGLN3 and the lack of other hypoxia responsive genes showing differential expression, it might initially seem that hypoxia is not a significant stress to animals native to high altitude environments. However, high altitude mice have higher hematocrits than low altitude mice, so we can be sure that these animals are responding physiologically to high altitude hypoxia. Differences in the the results of this study and previous hypoxia gene expression studies might reflect the differences between acclimatizing to hypoxia compared with having developed, lived, and perhaps adapted to hypoxia over multiple generations. Because most dramatic differences in gene expression likely occur during the acclimatization process, it seems feasible that fewer and different genes are expressed in animals fully adjusted to hypoxia than are expressed in animals in the process of acclimatizing. Factors other than hypoxia also might contribute to the variation in gene expression that we saw between low and high altitude mice. Unlike laboratory studies of hypoxia, high altitude is also characterized by relatively cooler climates, more dramatic seasonal changes, and many other environmental stressors that differ from low altitude. Undoubtedly, some variation in gene expression might be related to these climatic and other differences. Lastly, to our knowledge, this is the first study to characterize gene expression of wild mice at high altitude. Wild, outbred mice likely have different transcriptional responses to environmental stress than inbred laboratory mice. Whatever the reason for the lack of congruence with other hypoxia studies, this study indicates that laboratory gene expression experiments on acute hypoxia are poor predictors of

how natural populations cope with and survive in high altitude environments. So while it might be appropriate to use laboratory studies to explore genetic and physiological mechanisms, understanding what happens in nature will require studies of animals in their natural environments.

Differentially expressed genes belonged to several broad functional categories, including various metabolic processes; protein production, regulation and destruction; development and growth; cellular processes and signaling; DNA maintenance, replication and repair; and immune system processes. Most of these categories were equally represented by genes showing increased and decreased expression, and it is difficult to speculate about their potential biological significance. However, at high altitude, genes associated with cell proliferation, growth and development primarily showed decreased expression, and genes associated with the immune system showed increased expression. Furthermore, these functional categories were represented by multiple genes showing more than two fold differences in gene expression, indicating that these processes and their functional significance deserve further discussion.

*Genes associated with cell proliferation, growth, and development.*

All but one of the genes associated with cell proliferation, growth and development showed decreased expression at high altitude. Of these genes, the probesets representing alpha fetoprotein and inositol polyphosphate multikinase both showed greater than 4 fold decreases expression at high altitude. Both of these genes are involved in embryonic development, and in adults are associated with cell proliferation and fertility (Frederick et al., 2005; Gabant et al., 2002; Li et al., 2002). Decreased expression of these genes is consistent with other genes in the growth and development list, which are involved in cell proliferation, tissue repair, and organ development (Jaendling and McFarlane, 2010; Li et al., 2010) (Table 2). Furthermore, genes involved in cell signaling and regulations of cell proliferation also showed differential expression.

It is unclear why there is a trend in decreased gene transcription for growth and development in mice that are larger than their low altitude counterparts.

Genes related to growth, development and cell proliferation might be decreased in the La Paz mice because they were trapped during autumn. In general, mice reach their peak population sizes in autumn, and the populations decline through the cold months of winter (Berry and Bronson, 1992; Silver, 1995). This pattern might be followed in La Paz, Bolivia, which experiences seasonal fluctuations in climate, but not in Lima, where the climate and temperature is relatively stable throughout the year. These potential seasonal differences in population growth might be reflected in gene expression in two ways. First, adult mice might be decreasing reproductive effort in the La Paz fall climate. Second, decreased reproduction might lead to a different age structure in the population of mice in La Paz. Due to potentially different age structure, we might have captured older, larger mice in La Paz that were no longer growing.

While changing temperatures and seasons are known to effect both growth and reproduction, and the immune system, the stress of hypoxia at high altitude is another major unavoidable stress on mammalian physiology. The effect of hypoxia on development and growth rate is complicated, and might be context and tissue dependent. Exposure and acclimation to high altitude hypoxia often results in reduced body mass (Baze et al., 2010; Bozzini et al., 2005; Quintero et al., 2010; Westerterp and Kayser, 2006). Animals born at low or high altitudes, but raised at high altitude are smaller than their lowland counterparts (Hammond et al., 2002). The mechanisms behind these changes are equally cryptic. While hypoxia can lead to reduced cell proliferation and apoptosis, many of these effects might be counteracted by the actions of HIF, which is now appreciated for its proliferative and anti-apoptosis actions, particularly in cancer (Harris, 2002; Lee et al., 2004). Therefore hypoxia likely has some effect on the genes involved in growth, development and cell proliferation.

*Genes associated with immune responses.*

Mice living at high altitude had increased expression of various immune related genes. In particular, they increased expression of IgA, IgM, immunoglobulin light chain- $\kappa$ , and immunoglobulin joining chain, the genes necessary for the production of circulating IgM and IgA antibodies. Pentameric IgM comprises up to 30% of the circulating antibodies, and is known for its role in protecting vital organs from bacterial and viral invasions via strong activation of the complement system. The complement component 3 (C3), the most abundant of the complement system proteins, also showed increased expression at high altitude. This result indicates that the IgM, complement activation system might be more heavily used at high altitude than low altitude. The antibody IgA, particularly the dimeric version, is found mostly in mucosal membranes and secretions, where it acts to protect the lumen of the gut and the respiratory membranes from external pathogens (Janeway et al., 2005).

Because the majority of circulating IgM antibodies are natural antibodies, and a fair proportion of IgA antibodies are also natural antibodies, one hypothesis might be that the natural antibody system is being relied on more heavily at high altitude than at low altitude. Natural antibodies are produced by a subset of B cells (B-1 cells) that produce germ line coded variable regions of immunoglobulins. Unlike other antibodies, organisms are born with natural antibodies, which require no prior exposure to antigen or vaccination. These antibodies are considered to be part of the innate immune system, providing a first line of defense against invading pathogens (Baumgarth et al., 2005).

Because the animals were trapped from various locations within both cities, it is unlikely that the differences between high altitude and low altitude mice can be attributed to exposure to particular pathogens or their health status, unless such difference in pathogen exposure or health status are systematically associated with altitude. There is evidence that both hypoxia and

seasonal fluctuations can influence immunological profiles of mammals. Immune systems are also known to fluctuate with changing seasons (Nelson, 2004). In small mammals, winter months are often associated with more active and responsive immune systems, presumably to counter higher winter infection rates or immunosuppression brought on by cold associated stress. Moreover, these fluctuations are thought to be triggered by decreasing temperatures and day lengths (Martin et al., 2008; Nelson, 2004). When taken together with the decreased expression of growth and reproductive genes, these results are consistent with the idea that the immune system and reproductive system compete for limited resources. As the winter approaches and reproductive efforts decline, then perhaps these limited resources might be available for increased immune function (Martin et al., 2008). However, such an interpretation requires considerable caution. While there are seasonal fluctuations in La Paz and not Lima, these fluctuations are still relatively mild compared to more temperate climates. In addition, changes in day length at these latitudes are smaller than in more temperate climates. Moreover, some genes involved in signaling both cell proliferation and immune responses affect these processes in as opposing manner, thus indicating that regulatory tradeoffs might be mediating these relationships rather than resource tradeoffs. For example, phosphatidylethanolamine binding protein 1 is important in the mitotic cell cycle, while potentially being a negative regulator of NF $\kappa$ B, a proinflammatory cytokine (Klysik et al., 2008).

The immune system of high altitude mice might also be influenced by hypoxia. Hypoxia and the immune system share several transcriptional and hormonal networks, indicating that hypoxia may induce immunological responses. This relationship is well established with acute hypoxia via the activation of hypoxia inducible factor (HIF), a transcription factor now recognized to have proinflammatory effects (Gale and Maxwell, 2010; Hellwig-Burgel et al., 2005). And although the influence of chronic, systemic hypoxia on the immune system is less

well established than in acute, localized hypoxia, there is some evidence that chronic hypoxia and high altitude exposure influence the immunology of mammals (Baze et al., 2011; Chohan et al., 1975; Facco et al., 2005). Furthermore, the results of this study are consistent with our previous chronic hypoxia microarray study, which demonstrated increased expression of immunological genes in inbred mice exposed to 32 days of hypoxia (Baze et al., 2010). Why IgA and IgM genes are preferentially expressed is difficult to ascertain. Natural IgM antibodies play an important role in ischemia/reperfusion injury by binding to tissues damaged during the ischemic phase (Chan et al., 2004; Fleming, 2006). However, this type of hypoxia is physiologically different than that experienced at high altitudes. Whether or not this process has any relationship to lower levels of chronic, systemic hypoxia deserves further consideration.

Microarray analysis of gene expression in wild populations is an attractive tool for many ecologists and evolutionary biologists wishing to study how animals respond to environmental stress. In his study, we utilized natural populations of a model organism to address questions of how the high altitude environments affects gene expression profiles. Our results indicate that laboratory studies on similar stresses and on laboratory versions of the model organism might be poor predictors of what happens within natural populations in natural environments. However, despite the overall lack of correlation with previous gene expression studies, this experiment was consistent with previous microarray results that identified the immune system as an important to living in hypoxic environments (Baze et al., 2010). Further research into the immune system is specifically affected by hypoxia, and the consequences this has for the animal's immunological responses, deserves further research. This study also showed that house mice, like humans, might have evolved changes in expression of the EGLN family of genes that enable them to cope with high altitude. This result highlights the utility of wild populations of *Mus* species as model organisms for high altitude research and excellent model organisms for ecological and

evolutionary physiologists. While we were not able to distinguish differences in acclimation from differences in Darwinian adaptation, we hope that this study provides the impetus and hypotheses to support future studies of evolutionary adaptation to high altitude environments.

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**TABLES**

Table 1. Genes and primers selected for confirmatory qRT-PCR analysis

<b>Ref</b>			
<b>Sequence ID</b>	<b>Common Gene Name</b>	<b>Forward</b>	<b>Reverse</b>
NM_028133	EGL nine homolog 3 (C. elegans)	ATAAGAATTGGGACGCCAAGTT	CCAGACAGTCATAGCGTACCT
NM_152839	Immunoglobulin joining chain	TGACGACGAAGCGACCATTC	TTCAAAGGGACAACAATTCGGA
NM_025300	Mitochondrial ribosomal protein L15	GGAGAGGTGTGACCATCCAG	GCATCAAGTGCCCTAGACTTCT
NM_028176	Cytidine deaminase	GATCTTCTCTGGGTGCAACATAG	CCTGAAATCCTTGTACCCTTCG
NM_007643	CD36 antigen	ATGGGCTGTGATCGGAACTG	GTCTTCCAATAAGCATGTCTCC
NM_027184	Inositol polyphosphate multikinase	TGGAGCAAGACAACGGGTG	CTCCGCTTCCGGGATCTC
NM_007393	Beta actin (Control Gene)	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Table 2: Differentially expressed genes previously reported to respond to hypoxia.

Gene Title	Probeset ID	One way Adjusted P value	Nested P value	Fold Change	Previous Expression Changes	
Methylthioadenosine phosphorylase	1424425_a_at	0.0191	0.00	1.57	decrease	Baze et al. 2010
Dystroglycan 1	1426778_at	0.019	0.0037	1.18	increase	Li et al. 2010
EGL nine homolog 3 (C. elegans)	1418649_at	0.019	0.0059	-2.09	decrease	Aggarwal et al. 2010
Suppression of tumorigenicity 5	1428372_at	0.049	0.0111	1.62	decrease	Baze et al. 2010
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1425247_a_at	0.029	0.0052	2.49	decrease	Baze et al. 2010
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1425324_x_at	0.029	0.0053	3.18	decrease	Baze et al. 2010
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427870_x_at	0.034	0.0060	1.89	decrease	Baze et al. 2010
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427756_x_at	0.051	0.0090	2.04	decrease	Baze et al. 2010
Very low density lipoprotein receptor	1438258_at	0.029	0.0114	1.61	Increase	Baze et al. 2010

Table 3: Genes showing differential expression expression at high altitude, arranged by functional group

Gene Title	Probeset ID	One way Adjusted P value	Nested P value	Fold Change
<b>Immune responses</b>				
Chemokine (C-C motif) ligand 19	1449277_at	0.040	0.0075	1.51
CKLF-like MARVEL transmembrane domain containing 8	1427964_at	0.047	0.0318	1.45
Complement component 3	1423954_at	0.036	0.0266	1.19
<b>Histocompatibility 2, class II antigen E alpha</b>	<b>1422892_s_at</b>	<b>0.039</b>	<b>0.0325</b>	<b>5.23</b>
Immunoglobulin heavy chain 2 (serum IgA)	1429381_x_at	0.040	0.0078	1.40
Immunoglobulin heavy chain 2 (serum IgA)	1421653_a_at	0.030	0.0153	1.92
Immunoglobulin heavy chain 2 (serum IgA)	1425763_x_at	0.030	0.0271	1.88
<b>Immunoglobulin heavy chain 6 (heavy chain of IgM)</b>	<b>1425247_a_at</b>	<b>0.029</b>	<b>0.0052</b>	<b>2.49</b>
<b>Immunoglobulin heavy chain 6 (heavy chain of IgM)</b>	<b>1425324_x_at</b>	<b>0.029</b>	<b>0.0053</b>	<b>3.18</b>
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427870_x_at	0.034	0.0060	1.89
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427756_x_at	0.051	0.0090	2.04
<b>Immunoglobulin joining chain</b>	<b>1424305_at</b>	<b>0.031</b>	<b>0.0057</b>	<b>5.26</b>
<b>Immunoglobulin kappa chain, constant region</b>	<b>1427455_x_at</b>	<b>0.033</b>	<b>0.0059</b>	<b>5.90</b>
<b>Immunoglobulin kappa chain, constant region</b>	<b>1427660_x_at</b>	<b>0.040</b>	<b>0.0075</b>	<b>4.63</b>
N-terminal EF-hand calcium binding protein 1	1437156_at	0.009	0.058	-1.72
<b>Myelin protein zero-like 2</b>	<b>1416237_at</b>	<b>0.009</b>	<b>0.056</b>	<b>2.08</b>
<b>Growth, Development, and Cell Proliferation</b>				
<b>Alpha fetoprotein</b>	<b>1416645_a_at</b>	<b>0.029</b>	<b>0.0188</b>	<b>-7.20</b>
Alpha fetoprotein	1416646_at	0.040	0.0192	-1.91
<b>Inositol polyphosphate multikinase</b>	<b>1430031_at</b>	<b>0.040</b>	<b>0.0295</b>	<b>-4.62</b>
Motile sperm domain containing 3	1460452_at	0.020	0.0048	-1.20
Notch gene homolog 1 (Drosophila)	1418634_at	0.034	0.0061	1.34
Translin	1448516_at	0.034	0.0180	-1.31
<b>Cellular Processes and Signaling</b>				
Baculoviral IAP repeat-containing 4	1437533_at	0.003	0.0014	-1.34
Cytoplasmic FMR1 interacting protein 2	1428347_at	0.036	0.0064	1.39
Desmocollin 2	1421156_a_at	0.040	0.0086	1.34
<b>Dynein light chain Tctex-type 1</b>	<b>1428116_a_at</b>	<b>0.015</b>	<b>0.0037</b>	<b>-2.99</b>
<b>Dynein light chain Tctex-type 2</b>	<b>1453473_a_at</b>	<b>0.020</b>	<b>0.0039</b>	<b>-3.36</b>
Neuroblastoma ras oncogene	1422687_at	0.019	0.0035	1.72
RAP2B, member of RAS oncogene family	1448885_at	0.034	0.0164	1.62
Regulator of G-protein signaling 3	1425296_a_at	0.055	0.0095	1.36
RIKEN cDNA 1200009F10 gene	1429065_at	0.006	0.0018	-1.62
RIKEN cDNA 8430419L09 gene	1449936_at	0.036	0.0064	-1.46

*Cell signalling involving Immune responses and Cell proliferation*

Dystroglycan 1	1426778_at	0.019	0.0037	1.18
Phosphatidylethanolamine binding protein 1	1415950_a_at	0.003	0.0051	-2.03
Phosphatidylethanolamine binding protein 1	1438649_x_at	0.003	0.0031	-1.71
Prostaglandin E receptor 2 (subtype EP2)	1449310_at	0.029	0.0049	1.45
Schlafen 2	1450165_at	0.040	0.0110	1.29

**FIGURE LEGENDS**

Figure 1. Mean body mass of six populations of house mice, three from low altitude and three from high altitude. High altitude house mice were had significantly larger than low altitude mice ( $p < 0.032$ ).

Figure 2: Mean hematocrit for six populations of wild house mice, three from high altitude and three from low altitude. High altitude house mice had significantly greater hematocrits than low altitude mice ( $P < 0.004$ ).

Figure 3: Principal component analysis of the eighteen Affymetrix arrays. Points represent individual mice from Low (L)(red) or High (H) (blue) altitude, and from population A, B, or C within those elevations.

Figure 4: Confirmatory real-time qRT-PCR of 5 transcripts. Comparison between the expression values from the Affymetrix GeneChip Mouse Expression Set 430 and expression values from quantitative real time PCR resulted in a 0.82 correlation coefficient. Igj is immunoglobulin joining chain; Cda is cytidine deaminase; Egl3 is EGL nine homolog 3 (*C. elegans*), or HIF prolyl hydroxylase protein 3; Mrpl15 is mitochondrial ribosomal protein L15; Impk is inositol polyphosphate multikinase.

## FIGURES

Figure 1.

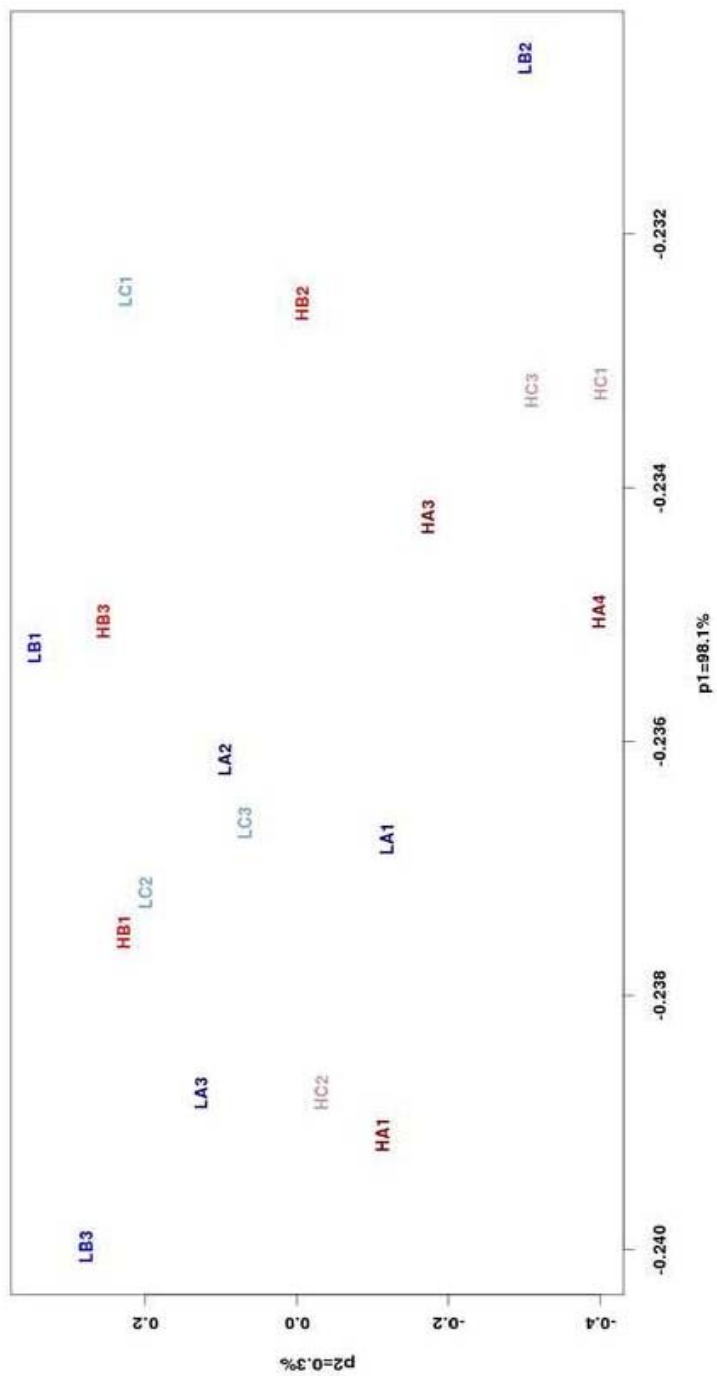


Figure 2.

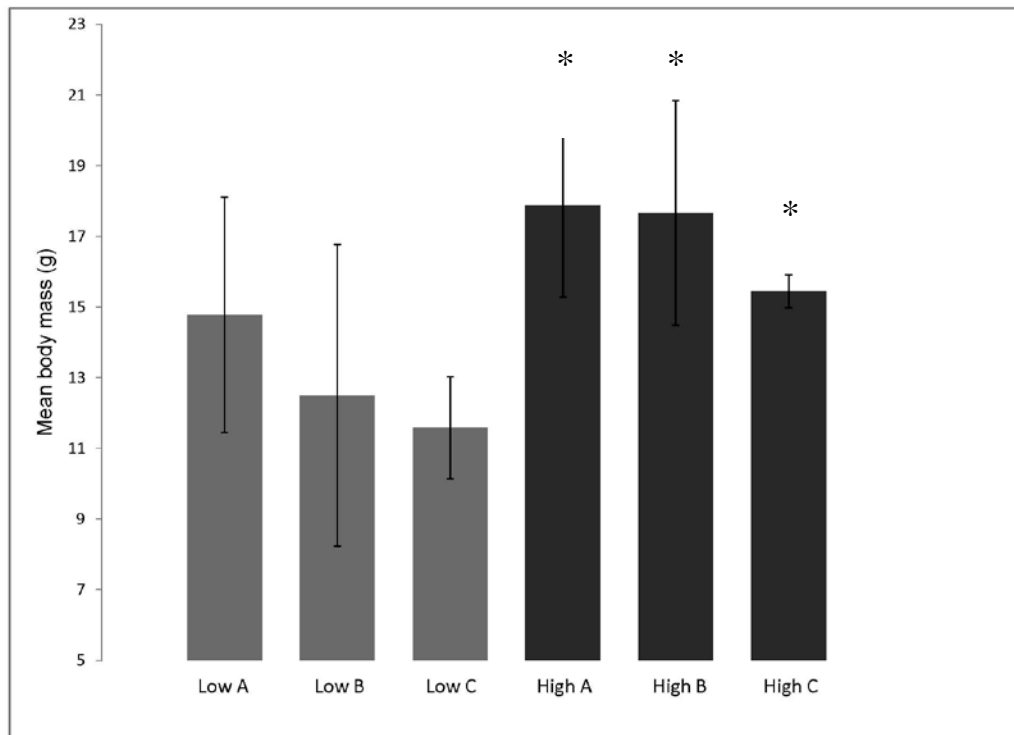


Figure 3.

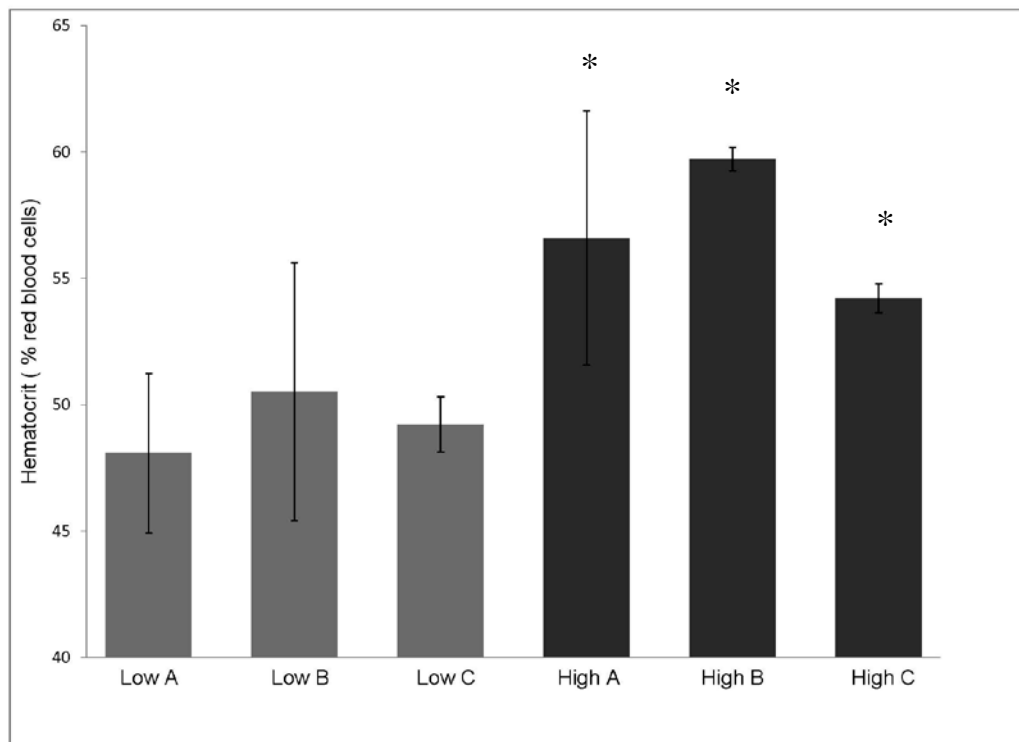
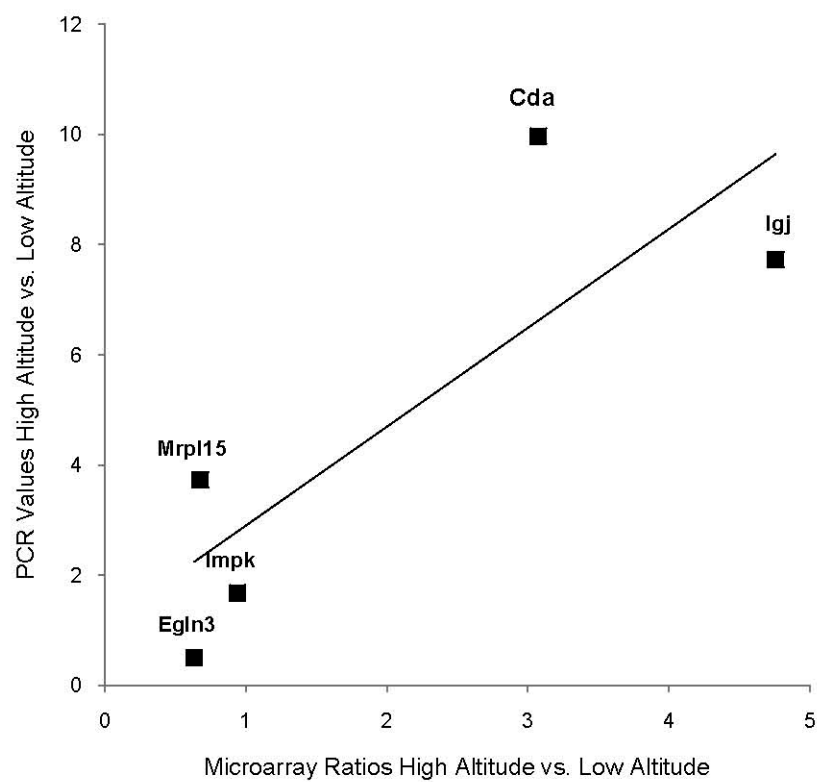


Figure 4.



**CHAPTER 3: CHRONIC HYPOXIA STIMULATES AN ENHANCED RESPONSE TO IMMUNE CHALLENGE WITHOUT EVIDENCE OF AN ENERGETIC TRADEOFF**

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**ABSTRACT**

There is broad interest in whether or not energy metabolism trades off with immune function. Under hypoxic conditions, maximum aerobic energy metabolism is limited, and other aspects of energy metabolism of animals may be altered. These energetic perturbations may affect immune function. While acute hypoxia appears to enhance certain immune responses, the effects of chronic hypoxia on immune function are largely unstudied. Herein we tested (i) whether chronic hypoxia affects immune function, and (ii) whether hypoxia affects the metabolic cost of immune function. First, flow cytometry was used to monitor the peripheral blood immunophenotype of mice over the course of 36 days of hypoxic exposure. Second, hypoxic and normoxic mice were subjected to an adaptive immune challenge via keyhole limpet hemocyanin (KLH) or to an innate immune challenge via lipopolysaccharide (LPS). The resting metabolic rates of mice in all immune challenge treatments were also measured. Although hypoxia had little effect on the peripheral blood immunophenotype, hypoxic mice challenged with KLH or LPS had enhanced immunological responses in the form of higher antibody titers or increased TNF- $\alpha$  production, respectively. Initially mice exposed to hypoxia had lower metabolic rates, but this response was transitory and resting metabolic rates were normal by the end of the experiment. Surprisingly, there was no effect of either immune challenge on resting metabolic rate, suggesting that mounting either the acute phase response or a humoral response is not as energetically expensive as previously thought. In addition, our results suggest immune responses to chronic and acute hypoxia are concordant. Both forms of hypoxia appear to stimulate both innate and adaptive immune responses.

## INTRODUCTION

Ecoimmunology is a growing field concerned with how variation in immune function affects organisms in different environmental and ecological contexts. Of particular interest are the potential indirect effects of the immune response on fitness via perceived tradeoffs with other physiological functions or life history traits (Lee, 2006; Lochmiller and Deerenberg, 2000; Schmid-Hempel, 2003; Schmid-Hempel and Ebert, 2003; Svensson, 1997). A common and often debated assumption is that the currency of these physiological tradeoffs is metabolic energy (Burness et al., 2010; Sheldon and Verhulst, 1996; Zysling et al., 2009). For this assumption to be true, the immune system must be energetically expensive to maintain and deploy such that it diverts energy away from other physiological processes. Attempts to demonstrate energetic costs of the immune system have yielded conflicting results (Demas et al., 1997; Mendes et al., 2006; Nilsson et al., 2007; Norris and Evans, 2000; Raberg et al., 1998; Scantlebury et al., 2007; Schubert et al., 2008; Svensson et al., 1998; Zysling et al., 2009). Perhaps factors other than energetic costs mediate the relationships between the immune system and other physiological processes.

An ecologically relevant method for examining physiological tradeoffs with the immune system is to compare the responses of environmentally stressed and unstressed animals (Burness et al., 2010; Ksiazek et al., 2003; Sandland and Minchella, 2003; Svensson et al., 1998). If coping with stress and meeting an immunological challenge are both energetically expensive, then one or both of the processes may be inhibited by the other. Indeed, there is much research demonstrating how thermal stress, nutritional stress, and psychological stress all lead to reduced immune function (Buehler et al., 2009; Cichon et al., 2002; Ksiazek et al., 2003; Kusumoto, 2009; Svensson et al., 1998). A caveat of these studies is that they do not directly address the question

of energy as the currency of the tradeoffs. Indeed, many stress responses may directly influence the immune system via endocrine mediators (Dhabhar et al., 1995; Martin, 2009). While it has been argued that endocrine mediators may be the proximate mechanisms driving energetic tradeoffs (Bourgeon et al., 2009; Demas et al., 2004; Martin et al., 2008b), this idea still depends on the immune system being energetically expensive. Factors other than energetic costs may be important in the evolution of immune function and its relationship to other functional systems (Ricklefs and Wikelski, 2002). Another, less discussed possibility is that the immune system and other physiological processes may share gene transcriptional networks. Transcriptional responses to hypoxia might also be important in mediating immune system effects.

Chronic, systemic hypoxia is commonly associated with high altitude environments. In mammals, ascending to high altitudes elicits a variety of physiological responses to maintain oxygen homeostasis, including polycythemia (increased hematocrit), increased production of 2,3 *bisphosphoglycerate*, pulmonary vasoconstriction, increased lung and liver mass, increased left ventricular mass, increased tidal volume and ventilation rate, increased capillary density, and anorexia with weight loss (Appenzeller et al., 2003; Beall, 2001; Bozzini et al., 2005; Hammond et al., 1999; Powell et al., 1998; Ward et al., 2000; Yu et al., 1999). Many of these responses have potential energetic costs, especially in light of the anorexia that hypoxia induces.

How hypoxia affects metabolic rates is a complex topic (Hayes, 1989a; Hayes, 1989b; Russell et al., 2008). Clearly, severe hypoxia reduces maximal metabolic rates compared to normoxia (Rosenmann and Morrison, 1975; Wehrlin and Hallen, 2006; Young et al., 1996). However, in this study we were not concerned with direct constraints on the upper limit to aerobic performance, but rather with the potential trade-offs with basal metabolic rates, which indicate the minimal cost of living (including basal costs of immune function) in a particular environment. Hypoxia leads some animals to down-regulate their body temperature and reduce

their basal metabolic rates (Gautier, 1996; Hochachka et al., 1996; Semenza et al., 1994; Steiner and Branco, 2002). Other studies suggest that hypoxia leads to increased basal metabolic rate or to increased rates of heat production in fasting animals (Butterfield et al., 1992; Han et al., 2003; Hayes, 1989). A further complication is that responses to acute and chronic hypoxia are potentially quite different, and while acute hypoxic responses are relatively well studied, responses to chronic hypoxia are much less well known. In any case, the fact that hypoxia and high altitude often are associated with changes in basal metabolic rate suggests that hypoxia is likely to be an environmental stressor that impacts energy metabolism.

Because both hypoxia and the immune system present potentially significant energetic - challenges to small mammals, we would expect that hypoxia might negatively affect immune function. In particular, reasonable hypotheses are that the more expensive innate inflammatory responses would be inhibited, and that the less expensive adaptive humoral immune response would be maintained (Lee, 2006; Martin et al., 2006; Tieleman et al., 2005). There is growing evidence that this may not be the case. For instance, several studies have reported that humans traveling to high altitudes have increased circulating leukocytes and decreased circulating CD4<sup>+</sup> T cells (Chohan and Singh, 1979; Chohan et al., 1975; Facco et al., 2005). These observations are consistent with the known the effects of acute hypoxia on the immune system within localized tissues, such as during tissue injury, inflammatory diseases, and cancer (Brouwer et al., 2009; Frantz et al., 2005; Lewis and Murdoch, 2005; Taylor, 2008; Walshe and D'Amore, 2008). In these cases, localized hypoxia leads to the recruitment and increased survival of macrophages, neutrophils and other granulocytes (Bosco et al., 2006; Burke et al., 2003; Murdoch et al., 2005; Thake et al., 2004; Walmsley et al., 2005b). Conversely, acute hypoxia appears to have a regulatory, anti-inflammatory effect on mature CD4<sup>+</sup> T-cells (Conforti et al., 2003).

That hypoxia may enhance or induce immunological responses seems contrary to the energetic tradeoff hypothesis. In this particular case, the apparent co-regulation of hypoxia and the immune system needs to be considered. Most of the hypoxia induced immune responses described above can be tied to regulation by either Hypoxia Inducible Factor (HIF) and/or Nuclear Factor Kappa Beta (NF $\kappa$ B). Both of these transcription factors have been identified as hypoxia sensitive, proinflammatory mediators (Michiels et al., 2002). While HIF was initially identified as a key modulator of most physiological responses to hypoxia (Greijer et al., 2005; Semenza, 2000; Yu et al., 1999), it is now appreciated as an inflammatory mediator via its proinflammatory effect on leukocytes and its interaction with NF $\kappa$ B (Cramer et al., 2003; Gale and Maxwell, 2010; Hellwig-Burgel et al., 2005; Zarembek and Malech, 2005; Zinkernagel et al., 2007). Conversely, NF $\kappa$ B is best known as the central transcription factor responsible for activating the acute phase inflammatory response via induction of key inflammatory cytokines, such as TNF- $\alpha$ . Recently NF $\kappa$ B has also been shown to be sensitive to hypoxic stimulation, independent of HIF (Chandel et al., 2000; Cummins et al., 2007; Rius et al., 2008; Taylor, 2008). Thus, it appears that through the activation of HIF and NF $\kappa$ B acute hypoxia may be a key signal to inflammatory responses (Taylor, 2008) and these immune responses may be part of the suite of physiological responses to hypoxia. While the significance of these transcriptional networks to chronic, systemic hypoxia is unclear, such co-regulatory relationships deserve consideration when looking at potential physiological tradeoffs in ecological immunology.

The energetic costs of the immune system and of responding to hypoxia deserve further investigation. While there is potential for these processes to compete for limited energetic resources, these interactions are trivial if either process does not demand a significant amount of metabolic energy. Indeed, mounting an immune response may not be as energetically expensive

as proposed—a topic which has been the subject of debate (Bourgeon et al., 2009; Klasing, 1998; Lochmiller and Deerenberg, 2000; Martin et al., 2008b).

To better understand how mammals may be affected by high altitude hypoxia, we investigated whether or not acclimating to chronic hypoxia alone alters the circulating immune system, and the effects of environmental hypoxia and immune challenges on metabolic rates. Specifically, we tested whether 1) chronic, systemic hypoxia affects the structure and function of the immune system; 2) immune responses have a significant energetic cost that is reflected in resting metabolic rate; and 3) there is evidence of an energetic tradeoff between acclimating to hypoxic stress and mounting an immune response.

## METHODS AND MATERIALS

### Environmental Treatments

Three experiments were performed using the same experimental apparatus on different mice. In the first experiment (later referred to as the time-series experiment), we measured circulating white blood cells in mice acclimating to hypoxia. In the second and third experiments, we measured the energy use of mice experiencing hypoxia, one of 2 immune challenges, or both hypoxia and an immune challenge simultaneously. In all instances, mice were housed for the duration of the experiments at one of two simulated altitudes. Two chambers simulated sea level by adding appropriate levels of oxygen to Reno ambient air (barometric pressure of 86.1 kPa,  $pO_2$  of 18 pKa). The final oxygen concentration of these chambers was approximately 24%, or an oxygen partial pressure ( $pO_2$ ) of 20.7 kPa. Two other chambers simulated the approximate hypoxic conditions found above 4000 m. The hypoxic chambers received Reno air mixed with nitrogen to achieve appropriate degrees of hypoxia. For the first study measuring white blood cells over time, the chambers were maintained at roughly 12% oxygen, or a  $pO_2$  of 10.4 kPa. If one adjusts for the dilutive effects of water vapor and the effect of the barometric pressure changes over altitude, the simulated altitude more closely approximates what mice would encounter at about 4500 m. For the second experiment, we adjusted the partial pressure of oxygen to 12.5% oxygen to match the level that was to be supplied to the respirometry chambers used to measure metabolic rates. This simulated altitude was closer to 4250 m. Details of methods for monitoring appropriate levels of oxygen and delivering oxygen to the chambers are described in Baze et al. (2010). Each chamber housed up to 12 mice individually in 30 x 8 x 12 cm standard rodent cages. Mice were provided with *ad libitum* food and water, and kept on a 12:12 LD cycle. Upon arrival in Reno from Charles River Laboratories (Hollister, CA), the C57BL/6 female mice were allowed to acclimate to the chambers for one week. All mice were 14 weeks of age at the

start of the experiment. All protocols were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

### **Responses to hypoxia over time**

To test how the immune system responded to systemic hypoxia, blood samples were taken from mice housed in hypoxic or normoxic environments on days 5, 15, 26 and 36. The total number of mice was 20, with each treatment represented by 10 mice, 5 in each chamber. On each of the 4 sampling days, we collected 140  $\mu$ l of blood from the mice to track the changes in immunophenotype over the course of hypoxic exposure. One 70  $\mu$ l microhematocrit tube was used to measure hematocrit. The second 70  $\mu$ l tube was analyzed with flow cytometry to detect changes in the white blood cell composition of the blood. Body masses of all animals were measured at each time point. Food consumption was estimated by weighing the food remaining after the 10 day period and subtracting that amount from the initial weight of food. Data from this experiment were analyzed in SAS using a split-plot ANOVA. Environmental treatment (hypoxia versus normoxia) was the main plot effect, time was the subplot effect factor, and the chamber was a random effect.

### **Hypoxia and immune challenge energetics**

The adaptive and the innate immune systems are thought to present different energetic demands on mammals. As such, we challenged hypoxic and normoxic mice with one of two inoculations to stimulate either the adaptive immune system or the innate immune system. The adaptive immune system was stimulated with injections of keyhole limpet hemocyanin (KLH), and resulting production of antibodies was used to assess the energetic cost of the humoral response to infection. The innate immune system was stimulated by injecting lipopolysaccharide (LPS). LPS is found in the cell walls of Gram negative bacteria, and it stimulates a strong acute

phase inflammatory response in mammals. Both the KLH and LPS experimental groups were each represented by 24 mice. The 24 mice were divided into 4 groups of 6 and assigned to one of the 4 treatments: Hypoxia/ inoculation; hypoxia/ Sham; normoxia/ inoculation and normoxia/Sham.

#### *KLH procedure*

Mice in the KLH treatment group were inoculated with 100  $\mu$ g KLH or Sham (saline) intraperitoneally in a volume of 200  $\mu$ l at the start of the experiment and placed in their appropriate environments. Five days after the inoculation, when B-cell proliferation should be at its peak, we measured their resting metabolic rates (see below). After the metabolic rates were measured, blood samples were taken via tail nick and collected in microhematocrit tubes, and animals were placed back in their environmental treatments. The blood was then centrifuged and hematocrit was measured as the percentage of packed red blood cells relative to the total blood volume. From these samples, plasma was extracted and frozen. At a later date, plasma levels of circulating KLH antibody were measured with an ELISA test (see below). The process was repeated again 9 days later, on day 14, when antibody production was likely at its peak. Twenty seven days after the initial immunization, the mice were immunized a second time to evaluate the metabolic demands of the “booster effect”, the secondary response to an antigen after having been primed by the first response. The same protocol described above was repeated again, with resting metabolic rate (RMR) and blood measurements taken 5 and 14 days after the second inoculation, or on days 32 and 41 of the experiment. All data were analyzed in SAS using a repeated measures split plot ANOVA. Environmental treatment was the mainplot effect (hypoxia versus normoxia), injection type (KLH or Sham) was the subplot factor, and chamber was a random effect. A group statement (which fits different error variances to the groups) was used to correct the problem of unequal variances between both KLH and Sham data, and between data

from the first injection versus data from the second injection.

#### *LPS Procedure*

Mice belonging to the LPS experimental group were housed in the hypoxic or normoxic chambers for 11 days prior to their first immunological challenge. At 11 days, when in the midst of physiologically acclimating to hypoxia, the mice were given an intraperitoneal injection of either 0.2 ml of 0.5 mg/kg LPS or a Sham of saline, and then they were placed in the metabolic chambers containing appropriate oxygen concentrations. Two hours after the injection, blood was collected in microhematocrit tubes via tail nicks, and then mice were weighed and immediately placed back in the metabolic chambers. The metabolic measurements began four hours after the injections, when the acute phase response should be highest. Animals were returned to their experimental environments after the metabolic measurements. After being exposed to LPS, there is a period of several days during which the animals are resistant to further inoculations, known as endotoxin tolerance. On day 27, when endotoxin tolerance was waning and animals were further acclimated to hypoxia, the entire inoculation and measurement process was repeated. From the blood samples collected at both time points, hematocrit was measured and plasma was collected and immediately frozen. At a later date, the amount of TNF- $\alpha$  present in the plasma was measured via a Luminex assay (see below). All data were log-transformed and analyzed in SAS using a Split plot ANOVA. Environmental treatment (hypoxia versus normoxia) was the main-plot effect, injection type (LPS or Sham) was the sub-plot factor, and chamber was a random effect.

#### **Flow cytometry**

Blood samples were collected in heparinized tubes and vials were quickly processed for analysis with flow cytometry. The focus of this assay was to count relative quantities of

circulating T cells, B cells, natural killer (NK) cells and polymorphonuclear leukocytes (PMNs). To this effect, the fluorochrome-labeled antibodies used for the assay were FITC-anti-mouse CD45 (eBioscience) to distinguish the population of CD45<sup>+</sup> leukocytes from the erythrocytes, PE-anti-mouse-CD3e (eBioscience) to distinguish T cells, PE-TxR-anti-mouse CD19 (Invitrogen) to distinguish B-cells, APC-anti mouse NK1.1 (eBioscience) to distinguish NK cells, and PC7-anti-mouse Gr-1 (eBioscience) to distinguish PMNs. Fifty microliters of this antibody cocktail was added to 50  $\mu$ l of blood and allowed to sit for 10 minutes. To this solution, 575  $\mu$ l of FACSlyse (BD Biosciences) was added after which the solution sat for another 10 minutes. Finally, 25  $\mu$ l of Streptavidin beads (Polysciences) were added to the solution. Antibody bound cells were examined with a four-color Beckman Coulter XL/MCL Flow Cytometer (Beckman Coulter, Hialeah, FL) in an identical manner as discussed in duPre et al. (2008). Signals from low angle forward light scatter (FS), orthogonal light scatter (SS) and four colors of fluorescence (FL1, FL2, FL3, FL4) were collected using a logarithmic amplification. The optical filters were set to collect ~525 nm (FL1 fluorescein), ~575 nm PE (FL2, PE) ~670 nm (FL3, PC5) and 740 nm (FL4, PC7). Data files were analyzed and median fluorescence intensities were determined with FlowJo Software (Tree Star, Inc., Ashland, OR.)

#### **Detection of KLH Antibodies with Enzyme-linked Immunosorbent Assay (ELISA)**

NUNC 96-well MaxiSorp polystyrene plates (Fisher Scientific, Fairlawn, NJ) were prepared by coating the plates with 50  $\mu$ l of 10  $\mu$ g /mL solution of KLH and incubating overnight at 4°C. The following morning, the plates were washed 3 times with PBS, then blocked by adding 200  $\mu$ l of PBS with 5% nonfat dry milk and allowed to incubate for 1 hour at 4°C. Plates were washed 3 times with PBS containing 0.05% Tween (PBS-T). Fifty microliters of mouse plasma diluted in PBS-T in 1:10, 1:100, 1:1000 and 1:10,000 ratios were added to the antigen-coated,

blocked plates and incubated at 4°C for 2 hours. After washing the plates 3 times with PBS-T, 50 µl of 1:10,000 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulins (IgA, IgG, IgM) was added to the plates and incubated at 4°C for 1 hour. After another 3 washes with PBS-T, 50 µl of freshly prepared TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD) was added to each well and incubated at room temperature for 30 minutes. Fifty microliters of 1N hydrochloric acid was added to the solution in each well. The optical density was determined at 450 nm using Spectra-Max micro-ELISA reader (Molecular Devices, Hercules, CA). For each sample, plots of mean optical density versus log plasma dilution were made in Microsoft Excel and a logarithmic curve fitted for each line [ $y = a \ln(x) + b$ ]. The y value was chosen as an optical density in the mid portion of the linear curves (e.g., 0.5 optical density units), and titer was defined as the reciprocal of the serum dilution (x value) corresponding to the y value.

#### **Detection of TNF- $\alpha$ with Luminex**

After extraction of blood samples from the LPS mice, plasma was stored in 25 µl aliquots in polypropylene microcentrifuge tubes at -80°C. Luminex binding assay was used to detect circulating levels of the cytokine TNF- $\alpha$ . The assay was carried out in duplicate for each sample according to manufacturer's directions using the Mouse TNF- $\alpha$  singleplex bead kit (Invitrogen, Carlsbad, CA) and Luminex 100 System (Luminex Corporation, Austin, TX). To correct issues with unequal variance, data were log-transformed and then analyzed with a split-plot ANOVA.

#### **Resting metabolic rate**

Resting metabolic rates were measured with a flow-through respirometry system identical to that used in Wone et al. (2009) with the exception of the supply air gases. Briefly, this was a 16

chamber open-circuit system that allowed up to 12 mice to be measured simultaneously, with the remaining 4 chambers left empty to measure baseline controls of incurrent air. The 590 ml chambers received dry mixed air at 200 ml per minute STP, regulated by upstream mass flow controllers (Sensirion, Zurich, Switzerland). Incurrent flow rates and the sampling order of the chambers were controlled with LabView software and a custom automated control system. To avoid the potential physiological response of animals responding to rapid changes in oxygen partial pressure, the metabolic rates of the mice were measured at the partial pressure in which they were acclimated. Therefore, the air supplied to the mass flow controllers came from tanks of mixed air. The normoxia groups were measured under normoxic conditions, and received mixed air of 24% oxygen and 76% nitrogen. Likewise, hypoxia groups were measured under the hypoxic conditions to which they were acclimated, and received mixed air of 12.5% oxygen and 87.5% nitrogen. Temperature of the chambers was maintained at 30°C, which is within the thermal neutral zone of *M. musculus* (Gaskill et al., 2009; Gordon, 1985). After exiting the chambers, the excurrent air was scrubbed of water and CO<sub>2</sub> with Drierite and Ascarite II, respectively, and then monitored with two Oxilla II dual channel oxygen analyzers. Because these oxygen analyzers were capable of measuring 4 chambers simultaneously, excurrent air going to the analyzers was switched every 15 minutes, such that each mouse was measured once per hour. Between measurements of each set of animals samples of baseline air were taken from the 4 empty control chambers. RMR of the mice was measured for a 4 hour period, giving us 4 separate 15 minute measurements from each mouse. These measurements were monitored at 1000 Hz, and the averages recorded every 5 seconds. Oxygen consumption (i.e., RMR) was calculated using the appropriate equation (Hill, 1972). Prior to the RMR measurements, mice were fasted for 12 hours and weighed to the nearest 0.01 gram. Measurements took place in the early portion of the light cycle. KLH mice were allowed to adjust to the chamber environment for an hour before

measurements began. Because LPS mice were being manipulated prior to the RMR measurements, they were placed in the metabolic chambers 4 hours prior to measurements both for adjusting to the metabolic chambers and for maintaining their oxygen environmental conditions during the manipulations. At the conclusion of RMR measurements, mice were weighed and returned to their treatment environments. For logistical reasons, normoxic mice were measured together in one group, and the hypoxic mice as another group the same time the following day. The lowest 5 minute metabolic rate of the 4 sampling periods was used as our final RMR measurement. Body mass is reported as the means of the before and after measurements.

## RESULTS

### Responses to hypoxia over time

#### *Body mass and growth rate.*

Initially, mice assigned to the hypoxic treatment were slightly but not significantly heavier than those assigned to the normoxic treatment. Hypoxic mice lost mass during the first 4 days of exposure to hypoxia, and by day 5 they were significantly smaller than normoxic mice ( $p < 0.001$ , Figure 1A). Hypoxic mice remained significantly smaller throughout the experiment ( $p < 0.0028$ ,  $F_{1,2} = 357$ ). The significant differences in mass and growth rate between hypoxic and normoxic mice all took place in the first few days when hypoxic mice lost mass. Over the first 5 days, growth rate differed significantly ( $p < 0.001$ ) as hypoxic mice lost body mass (-0.323 g/day) and normoxic mice gained body mass (0.112 g/day). Subsequently growth rates were not significantly different, and they were more similar in magnitude than during the first 4 days.

#### *Food consumption.*

Average daily food consumption was not statistically significantly different between the hypoxic mice and normoxic mice at any point in the experiment. This result was unaffected by whether or not body mass was included in the model as a covariate (Figure 1B).

#### *Hematocrit.*

Hematocrit of hypoxic mice was significantly different from normoxic mice by day 5, and remained significantly different through the experiment ( $p < 0.001$ ,  $F_{1,2} = 1894$ ). The hematocrit of hypoxic mice climbed to a mean of 76.6% by day 36 (Figure 1C).

#### *Flow Cytometry.*

The numbers of circulating immune cells were quantified via flow cytometry. The total number of CD45<sup>+</sup> (hematopoietic cells carrying the CD45<sup>+</sup> antigen) was expressed as number of cells per microliter of blood. B-cells, T-cells, NK cells, and PMNs were quantified as numbers

per microliter, as well as their frequency in the total number of white cells (CD45<sup>+</sup> cells) counted. The numbers per microliter yielded somewhat erratic results. These numbers were often inflated or diminished by high or low blood volumes used for flow cytometry. While there were a fair number of CD45<sup>+</sup> cells not identified on days 15 and 36, the expression of the cell types as their frequency of the total white blood cell count was much more consistent. Whether or not the data were analyzed in terms of cells per microliter or as a frequency of CD45<sup>+</sup> cells, no significant differences were found with regards to B cells, NK cells or PMN. However, on day 36 of hypoxia, hypoxic mice had significantly higher frequencies of T cells (45.6%) than normoxic mice (39.1%,  $p < 0.019$ ) (Table 1).

### **Hypoxia and Immune Challenge Energetics**

#### *KLH Experiment*

*Body Mass.* At the start of the experiment, mean body mass of the mice (measured in unfasted mice) was not significantly different across the treatments. Thereafter, body mass of the mice varied with the environmental treatment. Hypoxic mice were on average smaller than normoxic mice, but these differences were only significant on day 32 ( $p < 0.003$ ). Body mass did not vary significantly with respect to KLH or Sham injections (Figure 2A), and there was never a significant effect of the interaction of injection and environment ( $p$  values ranged from 0.56 to 0.95). For reasons that are unclear, all mice showed reductions in body mass during the first 14 days of living in the environmental chambers.

*Resting Metabolic Rate.* Inclusion or exclusion of body mass as a covariate in the analyses of metabolic rates yielded qualitative similar patterns in the results. The lack of significant correlation between body mass and metabolic rate is consistent with previous metabolic measurements in this strain of mouse (Johnston et al., 2007) (Figure 3). For simplicity,

we report only the results from analyses that included body mass as a covariate. Days 4, 14, and 41 each had one extreme outlier with high RMRs, while day 32 had two extreme outliers, one high and one low. Inclusion or exclusion of these outliers did not affect the outcome of the analyses, and we report values of the analyses with these outliers excluded. The mean resting metabolic rates of mice were significantly lower in hypoxic mice than normoxic mice on days 4 (0.4706 ml O<sub>2</sub> min<sup>-1</sup> and 0.6050 ml O<sub>2</sub> min<sup>-1</sup> respectively;  $p < 0.0306$ ,  $F_{1,2} = 31.24$ ) and 14 (0.4221 ml O<sub>2</sub> min<sup>-1</sup> and 0.5793 ml O<sub>2</sub> min<sup>-1</sup>, respectively;  $p < 0.0293$ ,  $F_{1,2} = 32.67$ ). By day 32, while hypoxic mice still had lower RMRs on average than normoxic mice (0.5799 ml O<sub>2</sub> min<sup>-1</sup> and 0.6947 ml O<sub>2</sub> min<sup>-1</sup>, respectively), the differences were no longer statistically significant. On day 41, resting metabolic rates were similar across the treatments, although hypoxic mean RMRs were still slightly lower than normoxic mean RMRs. RMR showed no consistent pattern with respect to KLH or Sham treatment, and the RMR between these groups were not significantly different on days 5, 14, or 32. Only on day 41 was there a significant difference between KLH and Sham mice, with Sham mice having slightly higher metabolic rates than KLH mice (0.6244 ml O<sub>2</sub> min<sup>-1</sup> and 0.5766 ml O<sub>2</sub> min<sup>-1</sup> respectively;  $p < 0.042$ ,  $F_{1,16} = 4.87$ ), a trend opposite of what would be predicted if mounting a humoral immune response were energetically expensive (Table 2). The effect of an interaction between type of injection and the oxygen environment (hypoxia or normoxia) was never significant, with  $p$  values ranging from 0.12 to 0.53.

*Hematocrit.* By day 5, hematocrit was significantly different between hypoxic and normoxic mice ( $p < 0.0184$ ,  $F_{1,2} = 52.95$ ), and it remained different throughout the experiment (Figure 2B). Mean hematocrits for normoxic mice were between 51 and 52% throughout the experiment. In hypoxic mice, the hematocrit climbed to 57% by day 5 and reached a maximum of 63% by day 32. Hematocrit did not vary significantly with respect to immunological treatments during days 5 and 41. However, during day 14, when B-cell antibody production was likely high,

there appeared to be a significant interaction effect ( $p < 0.015$ ,  $F_{1,16} = 7.4$ ). On day 14, hypoxic mice injected with KLH had lower hematocrits (61.3%) than hypoxic mice injected with Shams (63.2%) ( $p < 0.0018$ ), although the differences were biologically modest. Hematocrits remained relatively stable until day 32 with the difference between KLH and Sham mice still significantly different ( $p < 0.0463$ ). By day 41, while KLH mice still had slightly lower hematocrits than Sham mice, the differences were no longer significant (Figure 2B).

*Antibody Measurements.* ELISA was used to test for the presence of KLH antibody in the mouse serum samples. This test was performed to validate the efficacy of the injections and to monitor antibody production in response to KLH. All animals injected with KLH tested positive for the presence of KLH antibodies, and none of the Sham animals were positive for these antibodies. Further analysis of the data was affected by whether or not we included four large outliers (data points 2 fold or higher than the next highest data point). Inclusion of the outliers inflated error variances, reducing the ability to detect possibly significant treatment effects. The four outlier points that were excluded were 1 KLH and 1 Sham mouse from day 31, and 1 KLH and 1 Sham mouse from day 41, and thus were evenly distributed across treatments. The data and analyses reported hereafter exclude these four large outliers.

The presence of antibodies to KLH in mouse plasma samples were validated by day four (mean titers of 11.1 for KLH, 4.37 for Sham;  $p < 0.016$ ). Mean antibody production in the KLH injected mice increased by more than 3-fold by day 14 (mean titer of 33.42, Sham 7.03;  $p < 0.002$ ). After the second KLH injection, the experimental mice showed the typical secondary immunization or “booster” response. On day 32, four days after the second injection, mice showed a 100-fold increase in antibody production (mean KLH titer 3984, Sham titer 2.33  $p < 0.01$ ) over day 14. On day 41, 14 days after the second injection, the mean KLH titers increased by another 53% (6088 for KLH, 2.98 for Sham;  $p < 0.01$ ). A significant treatment by

environment interaction was detected ( $p < 0.013$ ,  $F_{1,63} = 6.65$ ), which was driven by the higher KLH titers of hypoxic mice. The mean ELISA titers were consistently higher in hypoxic mice injected with KLH over normoxic mice, and these differences were statistically significant across 4 measurements of the experiment ( $p < 0.013$ ) when data was analyzed as a repeated measures split plot ANOVA. In particular, these differences were significant on days 32 ( $p < 0.0067$ ) and 41 ( $p < 0.002$ ) (Figure 4) (Table 3).

### *LPS Experiment*

*Body Mass.* At the start of the experiment, the initial body masses of the mice were not significantly different between immune treatments or environments. On day 11 when LPS was first injected, hypoxic mice had a smaller mean body mass than normoxic mice (19.7 g and 21.2 g, respectively), but the difference was only marginally significant statistically ( $p < 0.059$ ). This pattern remained through day 27, when hypoxic mice were still significantly smaller than normoxic mice (20.2 g versus 22.6 g,  $p < 0.0013$ ). The body masses of mice assigned to the LPS treatment versus the Sham treatment were never significantly different (Figure 5A), nor was the interaction with injection type or with environment ( $p < 0.98$  and  $p < 0.26$  for days 11 and 27, respectively).

*RMR.* Inclusion or exclusion of body mass as a covariate in the analyses of metabolic rates yielded qualitative similar patterns in the results (Figure 6). For simplicity, we report only the results from analyses that included body mass as a covariate. The RMR of mice was only significantly different between normoxic and hypoxic mice at day 11. Hypoxic mice had significantly lower RMR ( $0.521 \text{ ml O}_2 \text{ min}^{-1}$ ) than normoxic mice ( $0.638 \text{ ml O}_2 \text{ min}^{-1}$ ) ( $p < 0.046$ ,  $F_{1,2} = 20.44$ ). This pattern was absent by day 27, when hypoxic and normoxic mice had very similar RMRs of  $0.581 \text{ ml O}_2 \text{ min}^{-1}$  and  $0.585 \text{ ml O}_2 \text{ min}^{-1}$  respectively ( $p < 0.927$ ,  $F_{1,2} = 0.01$ ).

While the mean RMRs of LPS mice were higher than those of the Sham mice on both days and within both treatments, these differences were small and not statistically significant (Table 4). The interaction of injection type with oxygen environment was not significant on either day ( $p < 0.82$  and  $p < 0.99$ , days 11 and 27 respectively).

*Hematocrit.* By day 11, hypoxic mice had significantly higher hematocrits than normoxic mice (63.5% RBC and 54.3% RBC, respectively,  $p < 0.0112$ ), and this pattern remained through day 27 (62.4% RBC and 51.1% RBC respectively;  $p < 0.005$ )(Figure 5B). Overall, LPS mice had significantly lower hematocrits than Sham mice ( $p < 0.001$  and  $p < 0.004$  for days 11 and 27, respectively). However, the injection of LPS was administered to the animals shortly before hematocrit measurements were taken. Because measurable changes in red blood cell production occur over longer periods of time (days to weeks), it is unlikely these differences reflect a response to LPS. The interaction of injection type with oxygen environment was not significant on either day ( $p < 0.12$  and  $p < 0.244$  for days 11 and 27, respectively).

*TNF- $\alpha$  Measurements.* To verify that the mice were mounting an inflammatory response to the LPS injections, Luminex assays were run on the plasma samples to detect the presence of the inflammatory cytokine TNF- $\alpha$ . All mice injected with LPS showed significantly higher values for TNF- $\alpha$  than Sham mice, demonstrating that they all responded to the injections ( $p < 0.001$  on both days 11 and 27). The lack of significant TNF- $\alpha$  levels in sham mice indicates that neither the injection nor the blood collecting processes led to systemic inflammatory responses in control mice. The TNF- $\alpha$  response was stronger in hypoxic mice than in normoxic mice on both days. On day 11, hypoxic mice injected with LPS had higher mean TNF- $\alpha$  (1242.7 pg/ $\mu$ l) levels than normoxic mice injected with LPS (832.4 pg/ $\mu$ l). Analysis of this log-transformed data revealed a marginally significant difference ( $p < 0.065$ ). However, when a normoxic group outlier (3 times the value of the next highest value) was removed from the dataset, the mean for the normoxic

group changed to 509.8 pg/ $\mu$ l, and the differences between the log transformed data were highly significant ( $p < 0.0039$ ). The interpretation that follows reflects the data without the outlier. On day 27, mean TNF- $\alpha$  level of hypoxic mice (341.9 pg/ $\mu$ l) was again higher than mean TNF- $\alpha$  of normoxic mice (96.75 pg/ $\mu$ l). Analysis of the log-transformed data confirmed that the difference was significant ( $p < 0.026$ ) (Table 5). The p values associated with the overall interaction of environment and injection type were not significant on either day. However, these interaction terms include the differences between Sham Normoxia and Sham Hypoxia mice. Because the sham mice did not respond with significant TNF- $\alpha$  levels, inclusion of Sham mice in the interaction terms likely renders the the interactions for the experiment not significant. The concentrations of TNF- $\alpha$  at day 11 were much higher than those at day 27, and this lowered response to LPS is likely due to the waning effects of endotoxin tolerance (Sanchez-Cantu et al., 1989)

## **DISCUSSION**

The purposes of the study were to 1) test whether or not hypoxia had a significant effect on the immune system, particularly the circulating milieu of leukocytes, and 2) examine the energetic costs of coping with hypoxic stress, immune stress, and both simultaneously. The results of these studies were somewhat surprising. While hypoxia did not appear to have a significant effect on the peripheral blood immunophenotype, it did appear to have a positive effect on the response of the immune system to immune challenges. Hypoxia led to a transient decrease in resting metabolic rate, but this decreased metabolic rate neither appeared to affect the immune system, nor did immune challenges appear to affect resting metabolic rate. The results of this experiment suggest that hypoxia has a complicated, positive relationship with the immune system that is not mediated by energetic costs.

### **Physiological effects of hypoxia**

Mice responded to hypoxia in several characteristic ways, indicating that they were indeed experiencing hypoxic stress. In all three experiments, hypoxic mice lost weight and remained smaller than normoxic mice, however, the effect was more pronounced in the time series study without any immune challenge. Interestingly, in the time series study, food consumption between hypoxic and normoxic mice was never significantly different, indicating that something other than anorexia was responsible for the loss in body mass. The starting body masses of both KLH and LPS mice were measured in unfasted mice, and are likely why initial measurements were higher than measurements on days 5 and 14. The mice we studied were relatively young and hence still growing. The large increase in body mass from day 14 to day 32 in the KLH mice likely reflects the more than two weeks of growth that had taken place between measurements. Body mass also dropped slightly between days 5 and 14 and between days 32 to

41. However, these slight drops in body mass are likely related to the slightly longer fasting periods that mice experienced on days 14 and 41 for logistical reasons. While loss in body mass is a common response to hypoxia, the exact mechanisms responsible for the change in body mass and the exact body components affected are still unclear (Quintero et al., 2010).

All mice responded to hypoxia with an increase in hematocrit. Again, the effect was more pronounced in the time series study, with hypoxic mice peaking at 76% red blood cells versus 63% for the KLH experiment, and 63.5 % in the LPS experiment. While mice from time series experiment experienced slightly more extreme hypoxia (12%) than mice from the KLH and LPS experiment (12.5%), it seems unlikely that this modest environmental difference fully explains such a large difference in hematocrit. We are unaware of other experimental factors that could account for these differences.

#### **Effect of hypoxia on the immune system**

The flow cytometry data indicate that hypoxia had little effect on the composition of peripheral blood leukocytes. The total numbers of leukocytes counted (CD45<sup>+</sup> cells) were never significantly different between the treatments, indicating that hypoxia does not lead to an increase in total leukocytes and that the extreme hematocrit of 76.6% did not have a negative effect on leukocyte production. The ratios of B cells, NK cells, and PMN cells across the entire experiment were within the normal range for mice, although the B-cell ratios were slightly lower than what is reported as average for 6 month old C57BL/6 mice (<http://phenom.jax.org>). These ratios were never significantly different between normoxic and hypoxic mice. The only significant variation found between the treatments was on day 36 when hypoxic mice had a significantly higher ratio of T-cells than normoxic mice. This result is the opposite of what would be predicted based on studies of humans at high altitudes and from studies of the effects of HIF on inflammation, in which hypoxia usually has a negative, regulatory effect on T-cell proliferation and function (Ben-

Shoshan et al., 2008; Conforti et al., 2003; Facco et al., 2005; Sitkovsky and Lukashev, 2005)

The reasons for this disparity are unclear, but perhaps they are related to differences in how small animals respond to chronic hypoxia.

While the flow cytometry data indicate that chronic hypoxia alone had little effect on the circulating immune system, hypoxia did affect how mice responded to immunological challenges. In response to both KLH immunization and LPS inoculation, hypoxic mice had more robust responses, particularly in response to the secondary injections. In KLH injected mice, ELISA titers increased dramatically over time and with secondary response, as expected. Overall, hypoxic mice had higher titers than normoxic mice, with these differences being highly significantly different on days 32 and 41. Data collected from the flow cytometry time series study indicated that this more robust immune response by hypoxic mice cannot be explained by a positive effect of hypoxia on peripheral B cell numbers.

The responses of mice in the LPS experiment followed a similar pattern to the KLH experiment. Data from the Luminex assay indicated the classic response to LPS over time, with a robust primary response in TNF- $\alpha$  production, and a less robust secondary response, which can be explained by endotoxin tolerance (Randow et al., 1995; Sanchez-Cantu et al., 1989). Like the KLH experiment, it is curious that the hypoxic mice had significantly higher circulating TNF- $\alpha$  levels on the second injection than normoxic mice. Again, it is unlikely that this response can be explained by alterations in numbers of circulating immune cells.

The more robust responses of hypoxic mice to KLH immunization and LPS inoculation are unexpected, but potentially very important results. One generally expects to find decreased immune responses during times of physiological stress. That hypoxia affects the immune system in such a way may have important implications for the evolution of animals colonizing high altitude, or for humans who travel to high altitudes. However, we recognize that caution is

warranted when interpreting these results. Large variation was inherent to these data sets, with responses to injections eliciting exponentially higher responses than their Sham counterparts. Furthermore, with low sample sizes, the data were sensitive to outliers, and exclusion of extreme outliers materially affected our interpretation of the ELISA and TNF- $\alpha$  analyses. Despite these pitfalls, the data indicate an interesting potential effect of hypoxia on the immune system, and this study suggests that larger scale, more detailed studies are warranted.

Transcriptional ties may explain the positive effect of hypoxia on immune responses (Safronova and Morita, 2010). As mentioned previously, through the induction of the HIF and NF $\kappa$ B, hypoxia has positive effects on the innate immune system via neutrophil migration, macrophage function, and the production of proinflammatory cytokines (Gale and Maxwell, 2010; Murdoch et al., 2005; Walmsley et al., 2005a). Recent evidence also suggests that hypoxia has a stimulatory affect on the maturation and differentiation of antigen presenting dendritic cells, which may aid antibody production (Rama et al., 2008; Spirig et al., 2010). These studies clearly establish a regulatory link between the immune system and acute, tissue level hypoxia. However, less is known about the effects of chronic, systemic hypoxia on the immune system. Evidence that the immune system is also affected by chronic, systemic hypoxia comes from studies at high altitude and of chronic obstructive pulmonary disease (Facco et al., 2005; McNicholas, 2009). Furthermore, despite the waning of HIF induction within hours of hypoxic exposure, studies on gene regulation and cytokine regulation suggest that the effect of hypoxia on the immune system persists over longer time scales than hours (Baze et al., 2010; Lam et al., 2008). Therefore, while chronic hypoxia has little effect on circulating immune cells, it may influence gene transcription and cytokine activity that affect immunological function.

Hypoxia-enhanced immune responses may also be related to endocrine system. For example, hypoxia temporarily increases circulating levels of melatonin. Melatonin generally leads to

increased immune function, especially in laboratory rodents (Frisch et al., 2004; Kaur et al., 2002). Similarly, hypoxia positively influences circulating levels of leptin, a hormone known to be a positive regulator of the immune system (Bernotiene et al., 2006; Fantuzzi and Faggioni, 2000; Grosfeld et al., 2002; Meissner et al., 2005; Shukla et al., 2005). Leptin is released by fat cells, and it is thought to be a signal to other physiological systems of the energy status of the animal. Leptin has been implicated as a mechanism by which energetic tradeoffs with the immune system occur. Typically, energy consuming processes deplete fat cells, decrease leptin levels, and therefore blunt the immune response (Demas and Sakaria, 2005). However, as in the case of hypoxia, a physiological stress that increases leptin release may actually positively influence the immune response. It is plausible that more robust immune responses in hypoxic animals occurred due to integration of regulatory relationships between hypoxia and the immune system. Understanding the effects of a physiological stress on the endocrine system may be important to consider when making predictions about relationships and tradeoffs with other physiological processes (Martin 2009).

### **Energetics of Hypoxia and Immune System**

Initially, hypoxic mice showed a decrease in RMR as compared to normoxic mice, consistent with the notion that hypoxia is a stressor that affects not only maximal aerobic output but also resting metabolism. However, for both the KLH and LPS experiments significant effects of hypoxia were only significantly different during the first two weeks of measurements. By days 27 and 34, RMRs were no longer statistically significantly different. In small mammals, hypoxia induced hypometabolism is often seen as an important adaptive strategy for saving energy in the face of decreased oxygen availability (Frappell et al., 1992; Frappell and Mortola, 1994; Gautier, 1996; Mortola, 2004; Singer, 2004). Previous studies on rats indicated that hypometabolic

response to hypoxia was transient (Olson Jr. and Dempsey, 1978), but until now, this trend has not been demonstrated in smaller animals such as mice. Our experiment suggests that the energy saving tactic of hypometabolism in small mammals is a transitory response. Perhaps metabolism returns to normal with the increase of hematocrit and other physiological responses to hypoxia that promote oxygen delivery.

A more surprising result is that injections of either the KLH or LPS immunological stimulant did not result in increased metabolic rates. The estimated cost of the humoral immune response is expected to be relatively low (Klasing, 1998; Martin et al., 2006; Raberg et al., 2002). In accordance with this idea, our results indicate that the humoral immune response induced by KLH does not lead to increased resting metabolic rates, even in the more proliferative secondary injection. That the mice were indeed mounting a humoral immune response was confirmed by ELISA. All mice injected with KLH not only showed positive ELISA titers, but also all demonstrated the characteristic secondary response to the second KLH injection. With that said, the most convincing evidence that the humoral immune response does not result in increased metabolic rate was the lack of correlation between the ELISA titers and RMR. If antibody production is associated with higher energy costs, then we would expect the hypoxic mice on day 41 to have the highest metabolic rates (Nilsson et al., 2007). In fact, on day 41, KLH mice had significantly lower RMR than Sham mice, with hypoxic KLH mice having an even lower mean RMR than normoxic KLH mice. These results are contrary to previous reports with C57Bl/6 mice, which estimate 30% rise in the metabolic rates of KLH injected mice (Demas et al., 1997). At this point, the reasons for our differing results are unclear, particularly given that both studies were conducted on the same strain of mice. However, studies on other species have also failed to find a significant relationship between the humoral immune response and metabolic rate

(Svensson et al., 1998) or a significant correlation between metabolic rate and the strength of an immune response (Nilsson et al., 2007).

In contrast to the adaptive immune response, the innate inflammatory response is expected to be quite expensive. The acute phase response is characterized by rapid inflammation, production and immigration of leukocytes, sickness behavior, and fever, and is estimated to increase metabolic rate by as much as 50% (Lochmiller and Deerenberg, 2000). Yet surprisingly there was no significant difference in metabolic rate in LPS treated mice versus Sham treated mice. Again, we know that the acute phase response was present in the LPS injected mice because all displayed the classic signs of sickness behavior, such as lethargy and piloerection (Hart, 1988; Lacosta et al., 1999), and all had high circulating levels of the inflammatory cytokine TNF- $\alpha$ . Possible differences in metabolic rate could have been masked if injection and blood collection also led to inflammation, thereby potentially increasing metabolic rates in all mice. However, because sham mice did not have elevated TNF- $\alpha$  levels, and all mice were within the normal range for BMR in C56BL/6 mice (Dinulescu et al., 1998), there is little evidence to support this idea. Another potential reason for not detecting significant differences in RMR between sham and LPS mice is that we did not measure and account for body temperature. Raising body temperature is estimated to increase basal metabolic rate by 10-15% per 1°C (Roe and Kinney, 1965), and hyperthermia likely accounts for a significant portion of energy expenditure during the acute phase response. The febrile response of C57BL/6 mice to LPS is dose and ambient temperature related, with the response starting to wane 4 hours after injection (Rudaya et al., 2005). Hence, it is possible that by the time of our RMR measurements, the febrile response was reduced or modest (Martin et al., 2008a). A lack of fever could account for a lack of difference in metabolic rate, especially because previously referenced estimates of the metabolic cost of the innate immune response included significant increases in body temperature

(Cooper et al., 1994; Fewell et al., 1991). Whatever the case, our data indicate that the inflammatory response itself is not as energetically expensive as previously predicted.

The metabolic costs of the immune system may have gone undetected due to metabolic compensation (Martin et al., 2008b). For the KLH experiment, compensation could occur by reducing energy and mass associated with other organ systems and diverting that energy to support B-cell proliferation and antibody production (Derting and Compton, 2003; Mendes et al. 2006). While we did not measure organ masses and cannot refute this hypothesis, there is little evidence to support it. Total body masses of the mice did not vary with respect to KLH challenge. With respect to the LPS-experiment, metabolic compensation could occur via altered behaviors of mice. An increase in metabolic rate induced by LPS treatment could be counteracted by lethargy (sickness behavior), anorexia and decreased energy turnover (Klasing, 2004). However, we do not think that this is a major factor in our study as metabolic rates were measured in fasting animals at rest – metabolic compensation by anorexia and inactivity are therefore unlikely.

One may argue that hypoxia is a unique physiological stress that may be an exception to usual relationships between environmental stress and the immune system. This view warrants consideration given that chronic systemic hypoxia is not a stress with which most mammals have evolved to cope. Indeed, the type of hypoxia that mammals are more accustomed to is that related to wounds and tissue damage. In this situation, it is quite understandable why the immune system would be *part* of the response, not a competing process. However, we maintain that this study is relevant to other ecological and environmental stresses. Firstly, the relationship between hypoxia and the immune system is an excellent example of how regulatory networks may complicate simplified interpretations of tradeoffs. Progress will likely require greater attention to understanding the nature of regulatory networks. Secondly, we found no lasting energetic cost of

hypoxia or of immune function. This result calls into question the putative metabolic, energetic costs of the immune system.

There is ample evidence of tradeoffs between the immune system and other life history traits, and there is strong evidence that the immune system is sometimes costly (Klasing, 2004; Lee, 2006; Martin et al., 2008b; Norris and Evans, 2000; Raberg et al., 2000). Exactly what those costs are deserves more consideration. For the energetic cost of the immune system to be significant enough to divert energy away from other systems, it ought also be significant enough to affect the metabolic rates of animals. That neither the humoral nor the innate immune responses elicited significant changes in metabolic rates should be cause for careful consideration by ecological immunologists. Indeed, there are many other costs of the immune system that may drive the ultimate evolution of tradeoffs between other systems, including increased exposure to oxygen free radicals, depletion of particular nutrients, increased susceptibility to autoimmunity, decreased embryo and gamete survival, and behavioral modification (Klasing, 1998; Long and Nanthakumar, 2004; Raberg et al., 1998; Ricklefs and Wikelski, 2002; Zuk and Stoehr, 2002). The immune system is arguably one of the most complicated physiological systems, and immunological phenotypes and responses are likely shaped by a multitude of sources of natural selection (Schmid-Hempel and Ebert, 2003). The field of ecological immunology seems poised to benefit from rapid progress as researchers combine mechanistic analyses with broad ecological and evolution perspectives, and keep an open mind about which currencies are most important physiologically.

**LIST OF SYMBOLS AND ABBREVIATIONS**

KLH	Keyhole limpet hemocyanin
LPS	Lipopolysaccharide
HIF	Hypoxia Inducible Factor
NF $\kappa$ B	Nuclear Factor Kappa Beta
PMN	Polymorphonuclear leukocytes
RMR	Resting Metabolic Rate
TNF- $\alpha$	Tumor Necrosis Factor alpha
ELISA	Enzyme-linked Immunosorbent Assay

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**TABLES**

Table 1: Circulating immunophenotypes of female mice exposed to normoxia or hypoxia for 36 days. Data expressed as percentage of total detected CD45+ cells. Not all CD45+ cells were positively identified as one of the four cell types, as indicated by the unidentified row, and this result was likely due to technical errors with flow cytometry. Days 15 and 36 had significant numbers of unidentified cells, which resulted in overall depression of percent cell counts. Despite the variation between days, normoxic and hypoxic mice never differed significantly from each other, except on day 36 when hypoxic mice had significantly higher T cell percentages than normoxic mice, as indicated in bold.

	Day 5		Day 15		Day 26		Day 36	
	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia
Bcell	38.0	41.8	22.8	25.5	42.9	41.9	28.8	28.6
Tcell	42.4	39.2	17.3	14.7	38.1	39.1	<b>39.1</b>	<b>45.6</b>
NKcell	2.13	1.67	6.12	4.74	2.87	3.11	1.77	1.33
PMN	14.7	13.9	8.98	5.00	5.62	4.45	8	9.98
Unidentified	2.85	3.48	44.8	50.05	10.6	11.5	22.4	14.5

Table 2. RMR values for the KLH study. Values are adjusted for body mass as a covariate, and are expressed in ml O<sub>2</sub> min<sup>-1</sup>. P-values for comparisons of interest are represented on the right.

	Normoxia		Hypoxia		P- values for mean differences	
	Sham	KLH	Sham	KLH	Hypoxia vs. Normoxia	KLH vs. Sham
Day 4	0.614	0.596	0.496	0.447	0.0306	0.190
Day 14	0.586	0.572	0.404	0.440	0.0293	0.482
Day 32	0.659	0.731	0.586	0.574	0.179	0.428
Day 41	0.634	0.608	0.615	0.549	0.290	0.042

Table 3: Mean ELISA titers for KLH plasma antibodies in mice injected with KLH or a saline Sham. P-values for comparisons of interest are represented on the right

	Normoxia		Hypoxia		P- values for mean differences	
	Sham	KLH	Sham	KLH	KLH Hypoxia vs. KLH Normoxia	KLH vs. Sham
Day 5	2.57	6.17	6.11	16.1	0.0082	0.0159
Day 14	6.21	41.9	7.86	44.8	0.1616	0.0002
Day 32	3.24	3706	1.42	5206	0.0067	< 0.001
Day 41	3.09	4398	2.83	7780	0.002	< 0.001

Table 4: RMR of mice in the LPS study. Values are adjusted for body mass as a covariate, and are expressed in ml O<sub>2</sub> min<sup>-1</sup>. P-values for comparisons of interest are represented on the right.

	Normoxia		Hypoxia		P-values for mean comparisons	
	Sham	LPS	Sham	LPS	Normoxia vs. hypoxia	Sham vs. LPS
Day 11	0.625	0.652	0.513	0.529	0.046	0.396
Day 27	0.567	0.603	0.563	0.599	0.927	0.355

Table 5: Luminex values for TNF- $\alpha$  concentrations of mice injected with LPS or a saline Sham. Values expressed in pg/ $\mu$ l. P-values for comparisons of interest are represented on the right.

	Normoxia		Hypoxia		P- values for Log <sub>10</sub> mean differences	
	Sham	LPS	Sham	LPS	LPS Hypoxia vs. LPS Normoxia	LPS vs. Sham
Day 11	8.16	509	8.41	1242	0.004	< 0.001
Day 27	33.7	96.8	43.3	342	0.026	<0.001

## FIGURE LEGENDS

Figure 1. Physiological responses of house mice to 36 days of hypoxia. A.) Mean body mass over time in normoxic and hypoxic conditions. Starting body masses were not significantly different on 0. By day 4, hypoxic mice dropped in mass and were significantly smaller than normoxic mice. They remained so for the rest of the study ( $p < 0.0028$ ). B.) Average daily food consumption of mice, measured at days 5,15,26,36, and adjusted for body mass as a covariate. Food consumption was never significantly different between hypoxic and normoxic mice. C.) Mean hematocrit, or percent red blood cells, over time in normoxic and hypoxic conditions. On all four days measured, hypoxic mice had significantly higher hematocrits than normoxic mice ( $p < 0.001$ ).

Figure 2. Physiological responses of mice to hypoxia versus normoxia and KLH versus Sham. A.) Mean body mass at each time point. Day 0 body mass was measured in unfasted animals immediately prior to the start of the experiment. The remaining body masses are the mean of pre and post RMR measurements in fasted animals. Body masses were not significantly different at the start of the experiment; by day 5 hypoxic mice were smaller than normoxic mice and they remained so throughout the course of the experiment, but this difference was only significant on day 32 ( $p < 0.003$ ). Body masses of KLH versus Sham mice within environmental treatments were never significantly different. B.) Mean hematocrit, or percent red blood cells at each of the four time points. On all four days measured, hypoxic mice had significantly higher hematocrits than normoxic mice ( $p < 0.018$ ). Under hypoxic conditions, hematocrit was significantly lower in KLH mice than Sham mice on day 14 ( $p < 0.0018$ ) and 32 ( $p < 0.046$ ). Within environments, otherwise, differences between KLH and Sham mice were not significantly different.

Figure 3. Scatter plot of individual body masses versus resting metabolic rates of mice in the KLH experiment. A.) Day 4, excluding a single high outlier for RMR of a Normoxia KLH mouse. B.) Day 14, excluding a single high outlier for RMR of a different Normoxia KLH mouse, C.) Day 32 excluding a single extreme high outlier for RMR of a Normoxia Sham mouse and an extreme low outlier for RMR of a Hypoxia KLH, D.) Day 41 excluding an extreme high outlier for a Hypoxia Sham mouse.

Figure 4. Mean KLH plasma antibody titers for mice injected with KLH. Brackets with asterisks indicate significant differences. A.) Titers after the first injection of KLH, on days 5 and 14 for both hypoxic and normoxic mice. Titers were significantly higher in hypoxic mice than normoxic mice on day 5. B.) Titers after the second injection of KLH on days 32 and 41 for hypoxic and normoxic mice. Hypoxic mice had significantly higher titers than normoxic mice on both days ( $p < 0.0067$  and  $p < 0.002$ , respectively).

Figure 5. Physiological responses of mice in the LPS experiment to hypoxia versus normoxia and KLH versus Sham. A.) Mean body masses at three times. Day 0 body mass were measured in unfasted animals immediately prior to the start of the experiment. The remaining body masses are the mean of pre and post RMR measurements in fasted animals. Initial body masses were not significantly different; however, hypoxic mice were smaller than normoxic mice on days 11 and 27 ( $p < 0.059$  and  $p < 0.0013$ , respectively). Within environments, mean body masses of LPS mice were not significantly different from those of Sham mice. B.) Mean hematocrit of mice on both measurement days. Hypoxic mice had significantly higher hematocrits than normoxic mice on days 11 and 27 ( $p < 0.011$  and  $p < 0.005$ , respectively). Within environments mean hematocrits of LPS mice were not significantly different from those of Sham mice.

Figure 6. Scatter plot of individual body masses versus resting metabolic rates of LPS mice. A.) day 10. B.) day 27. No data points were excluded from these data sets.

## FIGURES

Figure 1

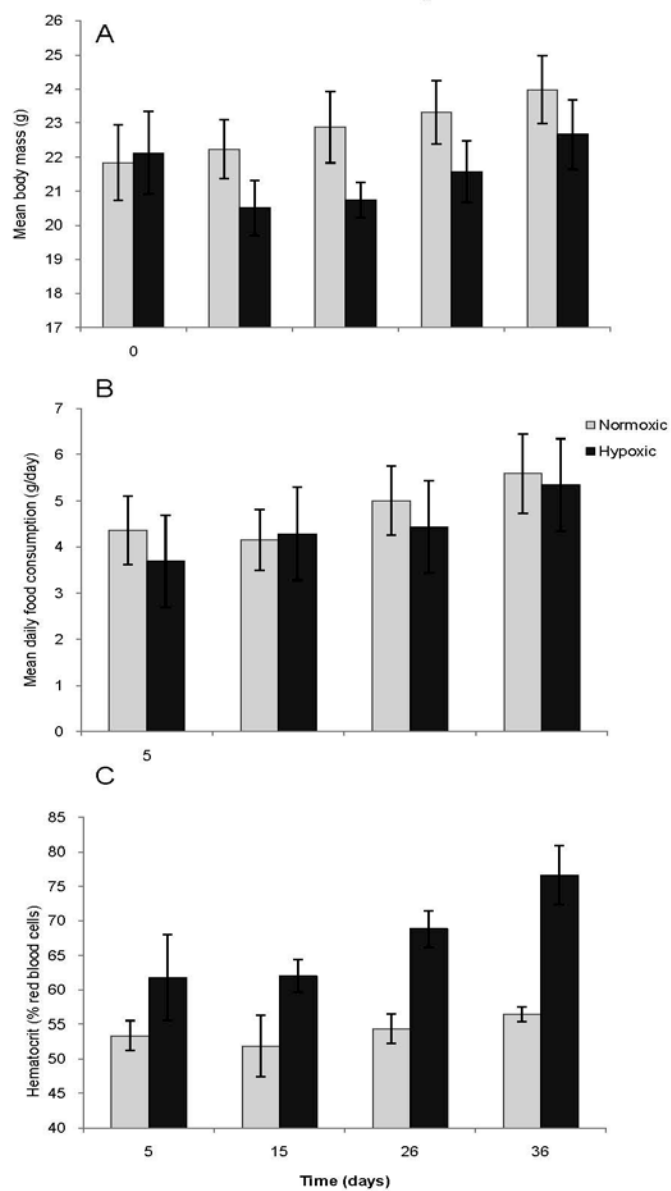


Figure 2

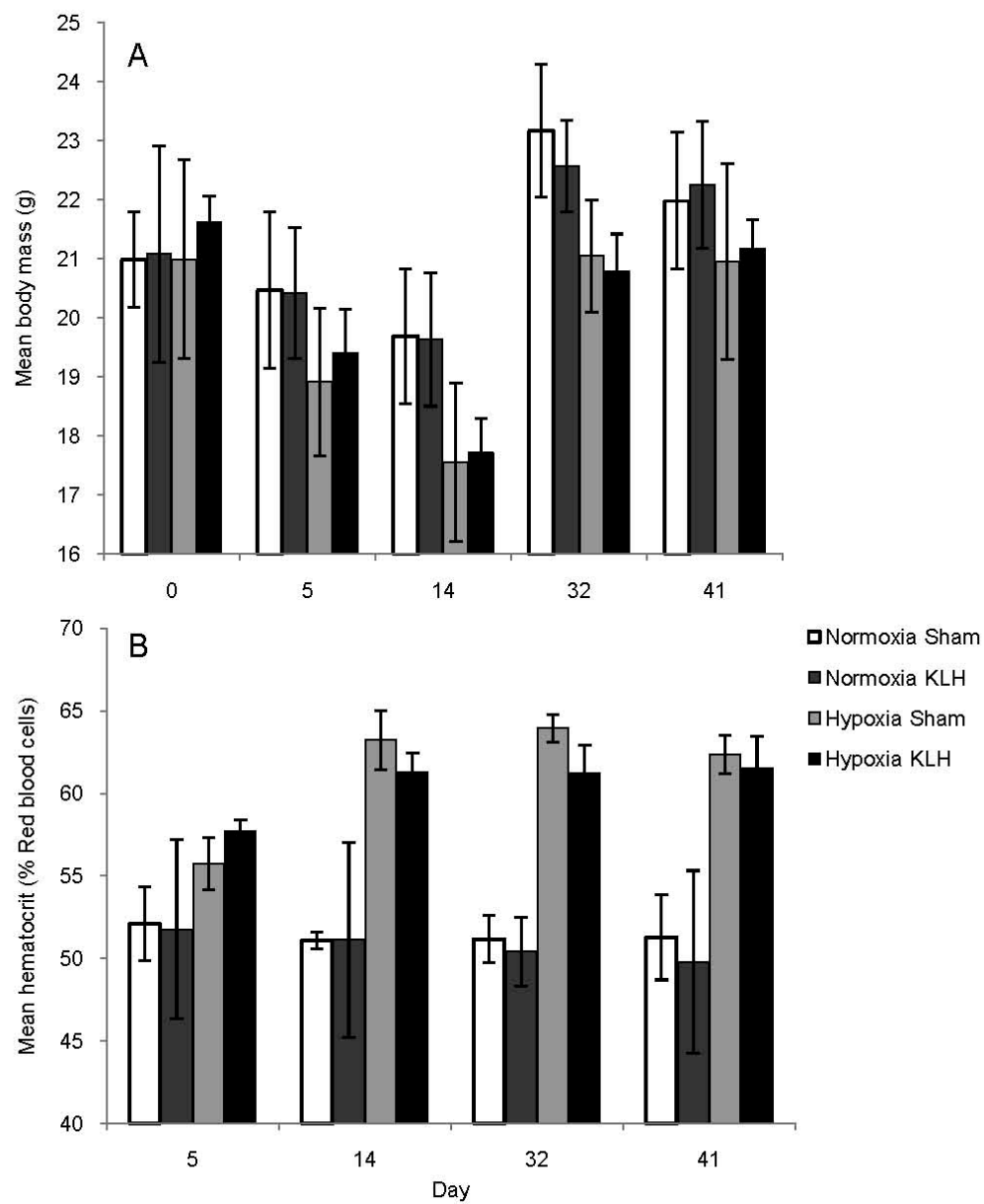


Figure 3

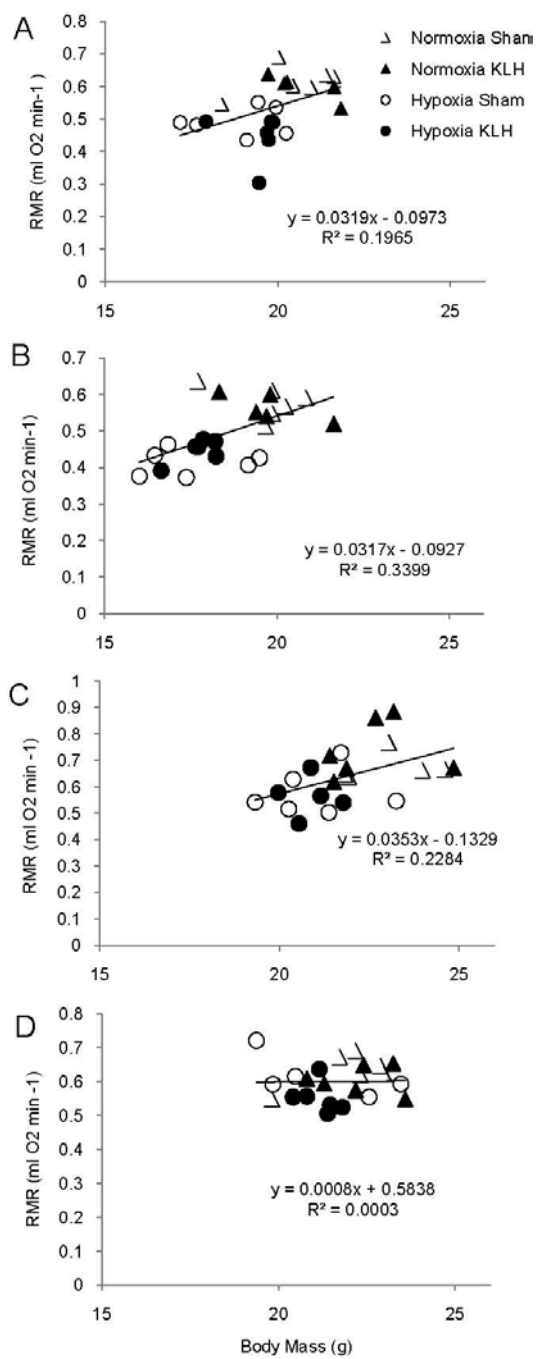


Figure 4.

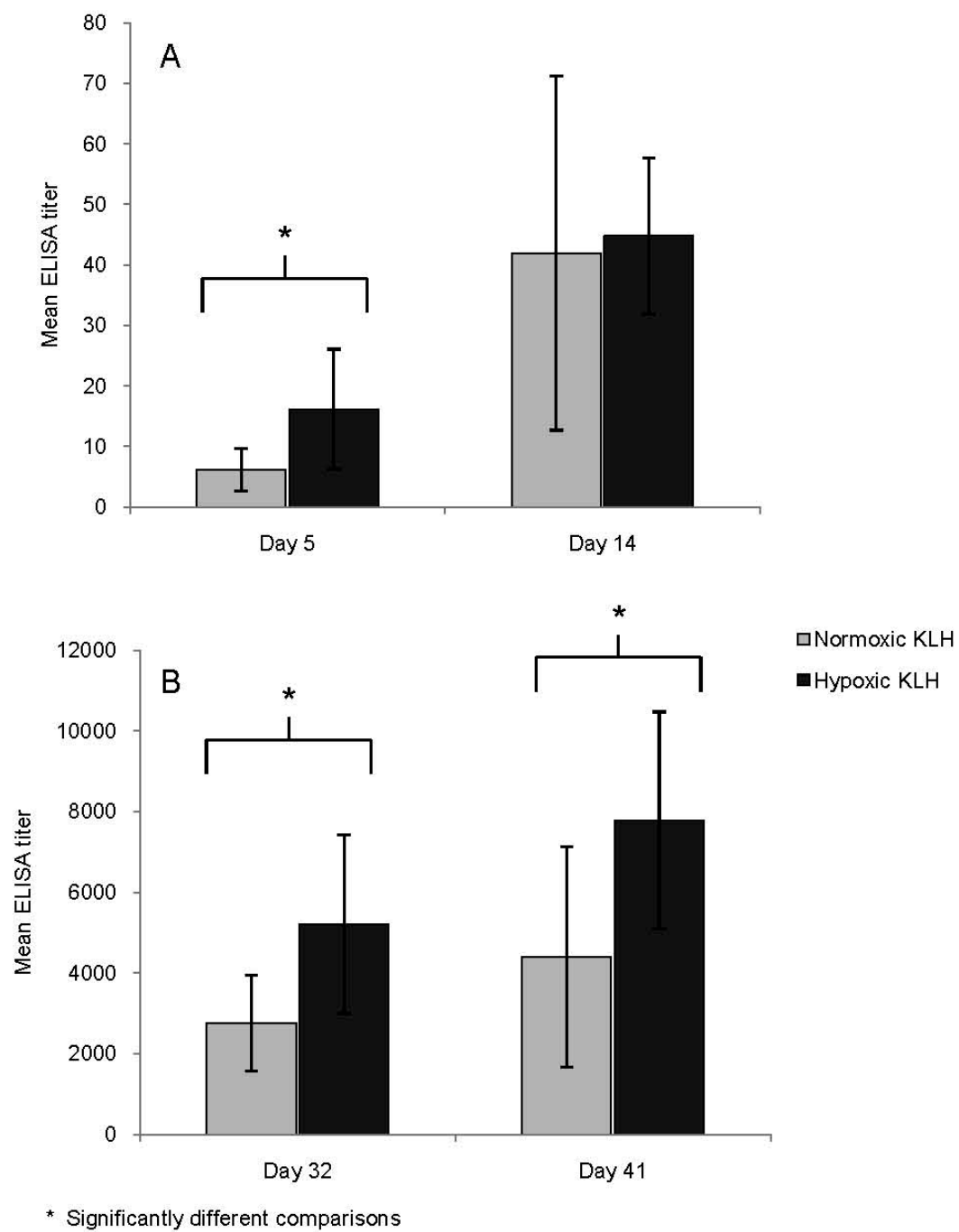


Figure 5.

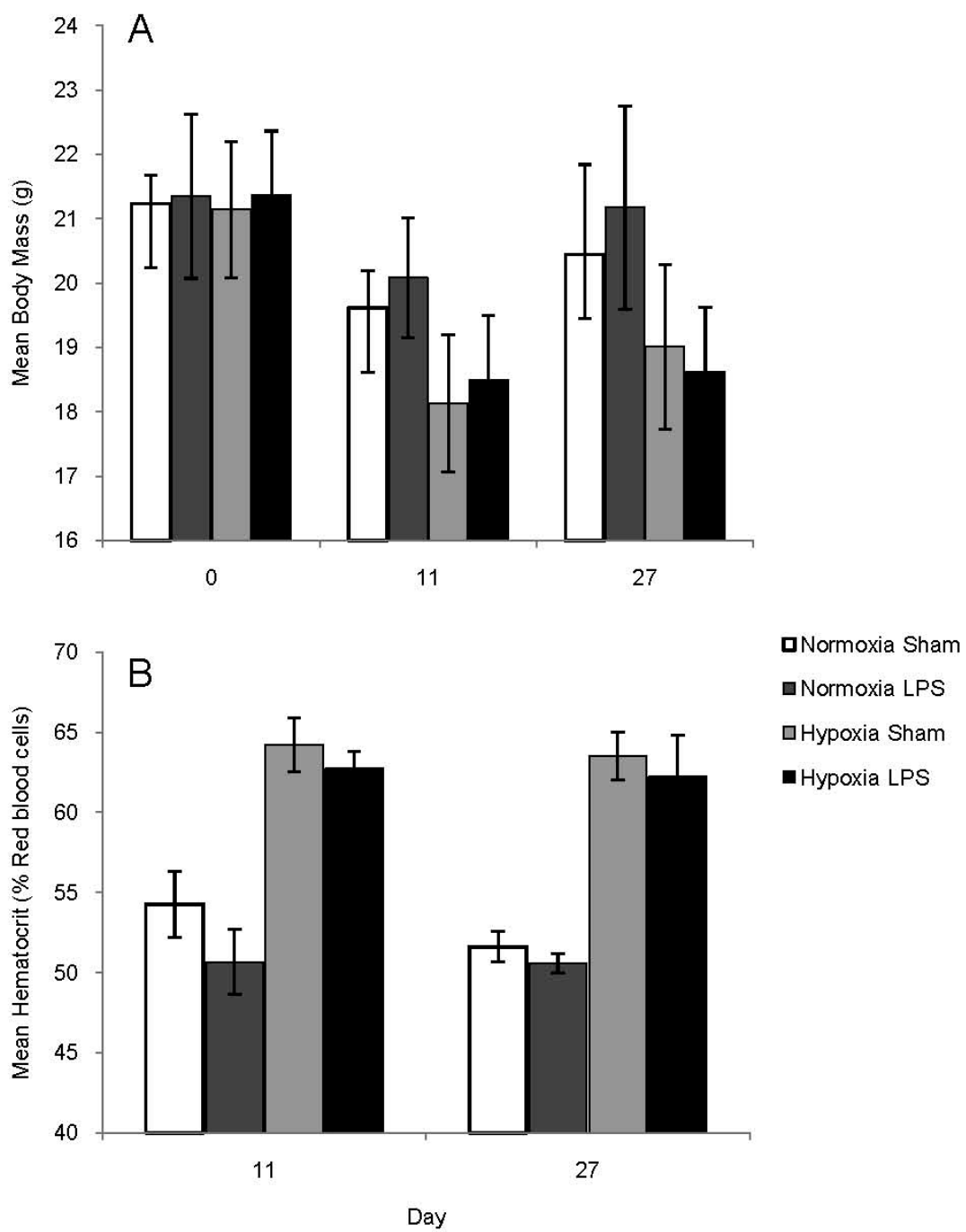
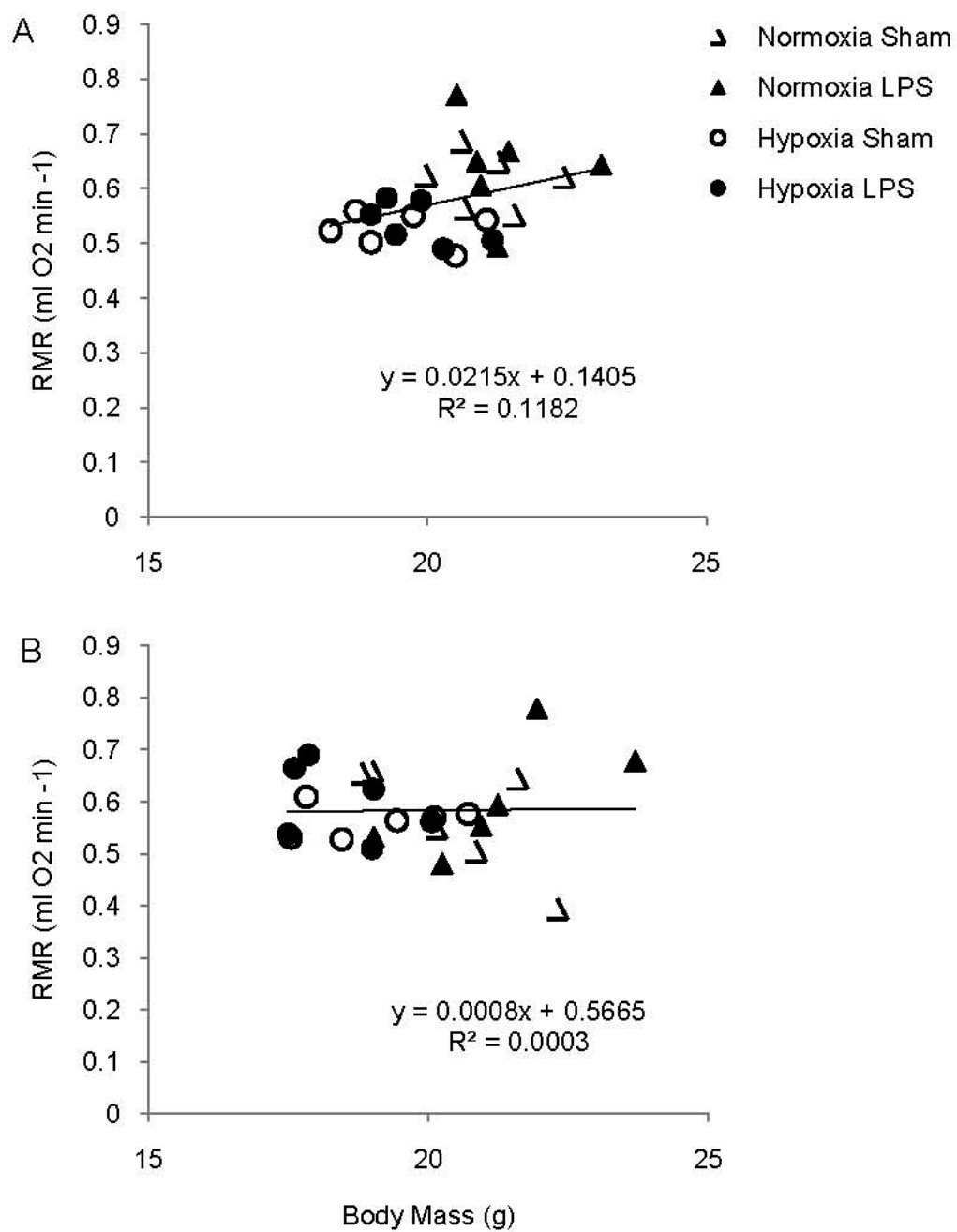


Figure 6.



## CONCLUSIONS AND RECOMMENDATIONS

How animals cope with, survive in, and adapt to extreme environments is of interest to physiologists, ecologists, and evolutionary biologists. The hypoxia stress of high altitude environments is an inescapable, repeatable stress across altitudes, and is particularly challenging for most terrestrial, non fossorial mammals. To further our understanding on how mammals physiologically cope with hypoxia, I utilized DNA microarrays to survey the gene expression profiles of house mice acclimated to hypoxia and of house mice resident at high altitudes. Information from these experiments was used to identify novel physiological processes that might be affected by high altitude hypoxia, yet have, thus far, received little attention. In particular, genes associated with the immune system showed increased in expression both in mice acclimated to hypoxia and in mice living at high altitude. A third study investigated how hypoxia influences the immune system, and how hypoxia and the immune system may affect the energetics of small mammals.

The first chapter described differences in gene expression in mammals acclimated to hypoxia versus controls. Responses in gene expression to acute or cellular hypoxia are well known, and it was expected that some of these same patterns would still be evident after 32 days of hypoxia. Specifically, we expected genes involved in the hypoxia inducible factor (HIF) pathway would show differential expression after chronic hypoxia as they do during acute hypoxia. However, of the 580 probesets showing differential expression between treatments, few were HIF-regulated genes. Rather, other genes important in the same functions as HIF-regulated genes, such as hematopoiesis and angiogenesis, showed differential expression. These results indicate that while similar functional processes are important in responding to acute versus chronic hypoxia, the specific genes involved in *responding* to hypoxia might be different from those involved in *maintaining* physiological acclimation to hypoxia. In particular, leptin receptor,

$\alpha$ -globin, and  $\beta$ -globin all had greater than 2 fold increased expression in hypoxic mice.

Circulating levels of the hormone leptin, a repressor of appetite, is known to increase in response to hypoxia. Several studies have attempted unsuccessfully to tie high levels of leptin to the anorexia displayed by mammals exposed to chronic hypoxia. However, few studies consider the expression of the receptor for leptin, and this receptor might also be subject to hypoxic regulation. Furthermore, while the genes for  $\alpha$ -globin and  $\beta$ -globin might be expected to be upregulated in animals with increased red blood cell production, this result has not previously been reported in the liver. Increased expression of  $\alpha$ -globin and  $\beta$ -globin is consistent with the increased hematocrit in the hypoxic mice, and it might be indicative of extramedullary hematopoiesis in the liver. Another response of mice to chronic hypoxia was the differential expression of genes involved in the immune system. While more recent studies have found that the immune system is regulated by HIF, the immune system was not previously appreciated as a significant factor in physiological acclimation to hypoxic environments.

In my second chapter I once again used DNA microarrays to profile the transcriptome of mice experiencing hypoxia. However, instead of using the highly defined system of acclimation to a single variable hypoxia in a highly inbred line, I investigated how altitude affects gene expression in nature. This experiment was conducted using wild (feral) mice trapped at high altitudes in La Paz, Bolivia, and low altitudes in Lima, Peru. This study resulted in few differentially expressed probesets (121 total). Most of the genes represented by these probesets were not reported previously to be related to hypoxia acclimation. However, consistent with studies showing that humans native to high altitude have decreased expression of EGLN1, the gene EGLN3 showed decreased expression in high altitude populations of mice. The ELGN family of genes (HIF prolyl hydroxylases) are negative regulators of HIF. The decreased expression of EGLN1 in humans is associated with an allelic variant in the gene, and carriers of

the low expression allele to have decreased susceptibility to HAPE (high altitude pulmonary edema). While there is evidence that low ELGN1 expression confers evolutionary adaptation to high altitude in humans, it is not certain that decreased expression of EGLN3 is the result of evolutionary adaptation in mice. However, my microarray study does support the finding that ELGN genes are important in coping with high altitude life. Also consistent with other studies on hypoxia gene expression, and the first chapter, was the differential expression of immune genes in high altitude (hypoxic) mice. More specifically, in high altitude mice there was increased expression of genes encoding the light, heavy and joining chains of immunoglobulin M (IgM) and immunoglobulin A (IgA). Because IgM and IgA are common isotypes of natural antibodies, it might be that natural antibodies are more important for mammalian immune systems than at low altitude, and this idea warrants further study.

Because the immune system genes were expressed differentially in both microarray experiments, the immune system might indeed be important in physiological responses to hypoxic environments. The third chapter explored the involvement of the immune system in responding to hypoxia, and the potential energetic consequences of responding to both hypoxia and immune challenges simultaneously. Counter to what would be predicted by the energetic tradeoff hypothesis proposed by some ecological immunologists, hypoxia enhanced responses to adaptive (KLH) and innate (LPS) immunological challenges. Furthermore, while hypoxia resulted in temporary decreases in resting metabolic rates, the immunological responses of mice to either KLH or LPS did not result in any changes in resting metabolic rate. That neither the humoral nor the innate immune responses elicited significant changes in metabolic rates should be cause for careful consideration by ecological immunologists. This result adds to the body evidence that calls into question the putative energetic tradeoff between stressors and immune function.

The first two chapters of this dissertation indicate that the effect of a stressor on gene expression can differ depending on the context in which the stress is experienced. For instance, changes in gene expression in response to acute hypoxia differed from gene expression in response to chronic hypoxia. Perhaps not surprisingly, but importantly, responses in laboratory settings may be poor predictors of responses in gene expression in natural populations. However, these studies support the idea that DNA microarrays are useful tools for exploring physiological responses to a stress that might not have previously been considered. In this case, the immune system was identified as a potentially important factor in responding to hypoxia in both the laboratory and naturally at high altitudes. To follow up on this finding, functional studies I described in my third chapter documented that immune responses are enhanced by exposure to hypoxia.

All three chapters identified new possible directions for research. From the first chapter, the increased expression of  $\alpha$ -globin and  $\beta$ -globin in the liver along with the extreme hematocrits measured in the mice might indicate that extramedullary hematopoiesis is taking place. While extramedullary hematopoiesis is common in several anemic diseases, this phenomenon has not been explored as a response to hypoxia. Future experiments on mice at extreme altitude or extreme hypoxia, where hematocrits reach as high as 75% or 80%, should focus on liver and spleen histology for the presence of extramedullary hematopoiesis. In addition, the mechanistic role that leptin receptor plays in the leptin pathway deserves further consideration. As stated previously, attempts to link hypoxia induced increases in circulating leptin to hypoxia induced anorexia have not produced conclusive results. However, if increased expression of the leptin receptor is included in the model of leptin/hypoxia induced anorexia, a better picture might emerge.

Results from the second chapter also highlight interesting lines for future research. In the wild mice I studied, the decreased expression of EGLN3 showed was parallel to the decreased expression of EGLN1 in humans native to high altitudes. In humans, decreased expression of this negative regulator of HIF has been associated with genetic adaptation to high altitude. This raises the interesting hypothesis of convergent evolution for decreased expression patterns of ELGN genes in mice and humans. To test this hypothesis, further investigation in mouse allelic variation and expression of EGLN genes is necessary, and it might identify EGLN genes as key to high altitude adaptation and evolution in mammals. Furthermore, if *Mus* species show similar evidence of adaptation to altitude, they would represent a new and useful model organism for evolution at high altitude. The discovery of increased expression of IgA and IgM genes also led to the hypothesis that natural antibodies may be important at high altitude. While it is not certain whether these differences in transcript abundance equate to differences in production of IgA and IgM antibodies, this result leads to the interesting hypothesis that high altitude mice may higher antibody levels than low altitude mice. Future experiments on high altitude mice should investigate the abundance of these antibody isotypes, should further establish whether or not they truly are natural antibodies, and perhaps explore the immunological functions of these mice at high altitude. That natural antibodies might be important to the ecological immunology of an animal has received little attention and deserves further consideration.

Finally, my microarray studies indicated that the immune system responds to both hypoxia and high altitude. The third chapter investigated this result more fully. While my third chapter demonstrated that the immune system is indeed affected by hypoxia, this relationship may not be based on managing the energetic demands that hypoxia and the immune system both impose on an animal. Rather, I present the hypothesis that shared regulatory networks might be more important in determining how physiological stress affects the immune system. If

mechanistic relationship between physiological stress and the immune system is based on shared regulatory networks, then our predictions of how stress influences the immune system (and *vice versa*) might be very different from the predictions made by an energetic tradeoff hypothesis. Therefore, understanding these mechanistic relationships between stress and immune responses is necessary for understanding the effects that they have on the physiological ecology of an animal. Furthermore, while conservation and utilization of energy might be an ultimate explanation of the co-regulation of stress and immune responses, my study demonstrated that the energetic demands of the immune system may not be as substantial as other researchers have predicted. Therefore, research into alternative ultimate explanations should be explored. These alternative explanations may include avoiding tissue damage, managing oxidative stress and damage, and minimizing risk of autoimmunity.

## APPENDIX A: SUPPLEMENTARY TABLES FOR CHAPTER 1 AND 2

### CHAPTER 1 SUPPLEMENTARY TABLE

Table 1: 580 probesets showing significant differential expression (Adjusted F- statistic  $p < 0.05$ ) to chronic, systemic hypoxia in mouse livers

Common Gene Name	Probeset ID	Log2 Signal Intensity 1400 m	Log2 Signal Intensity 3000 m	Log2 Signal Intensity 4000 m	Log2 Fold Change (4000 m versus 1400 m )	Adj F- pvalue
leptin receptor	1456156_at	3.47	3.61	6.52	3.05	0.002
leptin receptor	1425644_at	3.16	3.48	6.09	2.93	0.001
regulator of G-protein signaling 16	1426037_a_at	6.92	8.04	9.18	2.26	0.024
regulator of G-protein signaling 5	1420941_at	5.76	6.53	8.00	2.24	0.000
fatty acid binding protein 4, adipocyte	1417023_a_at	7.39	7.67	9.61	2.21	0.000
G protein-coupled receptor 98	1425314_at	6.90	7.58	9.10	2.19	0.000
regulator of G-protein signaling 16	1451452_a_at	7.13	8.15	9.20	2.07	0.027
---	1420942_s_at	6.44	7.04	8.45	2.01	0.000
cytochrome P450, family 4, subfamily a, polypeptide 14	1423257_at	9.53	9.72	11.50	1.97	0.009
hemoglobin alpha, adult chain 1	1417714_x_at	9.31	9.61	11.18	1.87	0.000
fatty acid binding protein 4, adipocyte	1451263_a_at	6.80	7.10	8.66	1.86	0.004
synuclein, alpha	1436853_a_at	3.94	3.93	5.79	1.86	0.000
predicted gene, ENSMUSG00000073738	1439816_at	3.87	3.93	5.71	1.84	0.000
regulator of G-protein signaling 5	1420940_x_at	6.05	6.59	7.88	1.84	0.000
apolipoprotein A-IV	1417761_at	8.00	8.32	9.80	1.80	0.005
cytochrome P450, family 17, subfamily a, polypeptide 1	1417017_at	6.29	6.45	8.09	1.80	0.005
ATP-binding cassette, sub-family D (ALD), member 2	1438431_at	4.37	4.64	6.14	1.76	0.005

major facilitator superfamily domain containing 2	1428223_at	6.62	7.39	8.34	1.73	0.019
ATP-binding cassette, sub-family D (ALD), member 2	1419748_at	4.85	4.95	6.55	1.71	0.008
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1416432_at	4.97	6.08	6.64	1.67	0.021
ubiquitin specific peptidase 2	1417168_a_at	7.70	8.93	9.35	1.64	0.013
Von Willebrand factor homolog	1435386_at	4.52	4.80	6.15	1.63	0.004
synuclein, alpha	1418493_a_at	4.30	4.38	5.87	1.58	0.010
cytochrome P450, family 39, subfamily a, polypeptide 1	1418780_at	6.96	7.49	8.53	1.58	0.002
---	1417466_at	9.15	9.80	10.72	1.57	0.000
regulator of G-protein signaling 3	1425701_a_at	6.16	6.21	7.67	1.51	0.019
flavin containing monooxygenase 2	1453435_a_at	4.72	4.73	6.21	1.49	0.006
flavin containing monooxygenase 2	1435459_at	5.86	5.82	7.31	1.45	0.007
CCR4 carbon catabolite repression 4-like ( <i>S. cerevisiae</i> )	1425837_a_at	6.21	7.65	7.66	1.45	0.046
suppressor of cytokine signaling 2	1449109_at	7.37	7.31	8.80	1.43	0.006
wee 1 homolog ( <i>S. pombe</i> )	1416774_at	5.75	6.53	7.18	1.43	0.025
RIKEN cDNA 9030619P08 gene	1443889_at	6.84	6.72	8.27	1.43	0.005
apolipoprotein A-IV	1436504_x_at	9.53	9.76	10.95	1.42	0.012
connective tissue growth factor	1416953_at	5.17	4.91	6.56	1.38	0.013
periostin, osteoblast specific factor	1423606_at	6.91	6.90	8.28	1.37	0.001
RIKEN cDNA 3732413I11 gene	1452769_at	8.12	8.69	9.48	1.36	0.005
meiosis expressed gene 1	1423410_at	4.29	4.50	5.65	1.36	0.015
RIKEN cDNA D630004K10 gene	1440840_at	5.25	5.72	6.60	1.35	0.002
serum deprivation response	1416779_at	5.56	5.68	6.89	1.33	0.012
FK506 binding protein 5	1416125_at	7.05	7.27	8.35	1.30	0.016
RIKEN cDNA 4930431B09 gene	1448021_at	6.21	6.46	7.50	1.29	0.010
lipin 1	1418288_at	8.71	9.40	9.99	1.28	0.009
desmoglein 2	1426153_a_at	4.59	5.13	5.87	1.27	0.013
C-type lectin domain family 14, member a	1419467_at	6.52	6.80	7.80	1.27	0.001
ubiquitin specific peptidase 2	1417169_at	8.00	9.12	9.27	1.27	0.018
serum deprivation response	1443832_s_at	5.12	5.37	6.38	1.27	0.003
junction adhesion molecule 2	1436568_at	5.11	5.31	6.37	1.26	0.007
lipin 1	1426516_a_at	8.94	9.64	10.20	1.26	0.007
ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1420408_a_at	3.92	4.38	5.18	1.25	0.010
vascular cell adhesion molecule 1	1448162_at	5.74	5.80	6.99	1.25	0.009
suppressor of cytokine signaling 2	1418507_s_at	7.31	7.02	8.54	1.24	0.037
vimentin	1456292_a_at	5.53	5.30	6.76	1.23	0.012
macrophage receptor with collagenous structure	1449498_at	5.55	5.59	6.73	1.18	0.040

Rap guanine nucleotide exchange factor (GEF) 4	1421622_a_at	9.28	9.92	10.44	1.16	0.015
---	1448607_at	4.77	5.06	5.93	1.15	0.009
very low density lipoprotein receptor	1434465_x_at	3.97	3.84	5.10	1.13	0.007
butyrylcholinesterase	1437863_at	5.63	5.79	6.76	1.13	0.042
ankyrin repeat and KH domain containing 1	1453023_at	9.52	10.13	10.63	1.11	0.019
coiled-coil domain containing 85B	1435589_at	6.07	6.35	7.18	1.11	0.002
Rap guanine nucleotide exchange factor (GEF) 5	1455137_at	5.89	6.28	7.00	1.11	0.009
FK506 binding protein 5	1448231_at	6.56	6.89	7.66	1.11	0.011
EGF, latrophilin seven transmembrane domain containing 1	1418059_at	5.30	5.65	6.40	1.10	0.002
thyrotroph embryonic factor	1450184_s_at	6.67	7.49	7.76	1.09	0.022
growth arrest and DNA-damage-inducible 45 alpha	1449519_at	6.31	6.44	7.39	1.08	0.010
tumor necrosis factor receptor superfamily, member 19	1448147_at	5.84	5.96	6.92	1.08	0.005
tissue inhibitor of metalloproteinase 3	1449335_at	6.36	6.75	7.42	1.06	0.009
copine II	1424831_at	6.27	6.66	7.33	1.05	0.007
RIKEN cDNA 2010003K11 gene	1424626_at	7.03	6.93	8.07	1.04	0.025
Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 7	1438617_at	9.24	9.25	10.28	1.03	0.022
UDP-glucose ceramide glucosyltransferase	1421269_at	6.00	6.37	7.03	1.03	0.031
pre-B-cell colony-enhancing factor 1	1417190_at	8.17	8.59	9.19	1.02	0.017
tissue inhibitor of metalloproteinase 3	1419089_at	5.37	5.56	6.39	1.02	0.013
ST3 beta-galactoside alpha-2,3-sialyltransferase 5	1460241_a_at	8.30	8.65	9.31	1.01	0.010
---	1455320_at	7.40	7.78	8.41	1.01	0.032
B-cell translocation gene 3 /// predicted gene, EG654432	1449007_at	7.65	7.84	8.64	0.99	0.009
CD24a antigen	1416034_at	4.63	4.59	5.62	0.99	0.011
N-acylsphingosine amidohydrolase 3-like	1451355_at	6.56	6.76	7.55	0.99	0.003
peptidylprolyl isomerase C	1416498_at	4.97	5.26	5.95	0.98	0.005
CD38 antigen	1450136_at	5.01	5.30	5.99	0.98	0.002
glucokinase	1419146_a_at	7.91	7.79	8.89	0.98	0.009
coatamer protein complex, subunit gamma 2, antisense 2	1427320_at	4.06	4.11	5.04	0.98	0.014
vimentin	1438118_x_at	7.02	7.01	7.99	0.98	0.017
ring finger protein 186	1424794_at	7.63	7.70	8.60	0.97	0.001
coenzyme Q10 homolog B (S. cerevisiae)	1460510_a_at	8.96	9.66	9.93	0.97	0.023
heme oxygenase (decycling) 1	1448239_at	6.94	6.80	7.91	0.97	0.022
ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417403_at	8.93	9.02	9.89	0.97	0.016
neuregulin 4	1421681_at	5.10	5.54	6.06	0.96	0.028
vimentin	1450641_at	7.26	7.32	8.22	0.96	0.009

glial cell line derived neurotrophic factor family receptor alpha 1	1421973_at	7.41	7.82	8.37	0.95	0.005
expressed sequence AI314180	1439127_at	5.91	6.22	6.86	0.95	0.033
ankyrin repeat domain 37	1436538_at	4.43	4.29	5.38	0.95	0.007
platelet/endothelial cell adhesion molecule 1	1421287_a_at	5.27	5.44	6.21	0.95	0.019
sorting nexin 13	1444141_at	4.69	4.87	5.63	0.94	0.007
GPI-anchored HDL-binding protein 1	1453022_at	8.47	8.70	9.41	0.93	0.003
Atpase, class VI, type 11C	1442367_at	7.19	7.42	8.13	0.93	0.006
UDP-glucose ceramide glucosyltransferase	1421268_at	7.20	7.49	8.13	0.93	0.022
Rho-related BTB domain containing 1	1429206_at	7.97	8.47	8.89	0.92	0.019
tetraspanin 4	1448276_at	8.91	9.15	9.83	0.92	0.007
elastase 2A	1448281_a_at	5.12	5.55	6.04	0.92	0.036
neuropilin 2	1435349_at	5.92	6.13	6.84	0.92	0.007
coenzyme Q10 homolog B (S. cerevisiae)	1428487_s_at	7.83	8.65	8.74	0.91	0.035
coatomer protein complex, subunit gamma 2, antisense 2	1427123_s_at	4.78	5.06	5.69	0.91	0.029
CCAAT/enhancer binding protein (C/EBP), delta	1423233_at	6.48	6.81	7.39	0.91	0.022
intercellular adhesion molecule 2	1448862_at	5.29	5.54	6.19	0.90	0.011
regulator of G-protein signaling 4	1416286_at	3.64	3.87	4.55	0.90	0.024
transmembrane protein 2	1451458_at	5.78	5.93	6.67	0.89	0.022
serum deprivation response	1416778_at	7.07	7.23	7.95	0.88	0.005
leukocyte cell derived chemotaxin 1	1460258_at	7.74	8.04	8.62	0.88	0.022
cytotoxic T lymphocyte-associated protein 2 alpha ///	1416811_s_at	5.35	5.46	6.22	0.87	0.017
cytotoxic T lymphocyte-associated protein 2 beta						
serine (or cysteine) peptidase inhibitor, clade A, member 3G	1424923_at	5.96	6.16	6.82	0.86	0.035
procollagen, type III, alpha 1	1427883_a_at	8.10	8.08	8.96	0.86	0.013
immediate early response 3	1419647_a_at	4.86	4.57	5.71	0.86	0.035
caveolin 2	1417327_at	5.02	5.11	5.87	0.85	0.044
glucokinase	1425303_at	7.58	7.40	8.43	0.85	0.010
wingless-related MMTV integration site 2	1449425_at	5.96	6.26	6.80	0.85	0.013
ELOVL family member 6, elongation of long chain fatty acids (yeast)	1417404_at	9.49	9.49	10.33	0.84	0.024
tissue factor pathway inhibitor	1452432_at	4.41	4.70	5.25	0.84	0.020
ectonucleotide pyrophosphatase/phosphodiesterase 1	1419276_at	7.25	7.42	8.09	0.84	0.030
solute carrier family 44, member 2	1438559_x_at	5.28	5.47	6.11	0.83	0.047
C-type lectin domain family 4, member n	1425951_a_at	3.82	3.85	4.66	0.83	0.004
CD68 antigen	1449164_at	6.06	6.13	6.89	0.83	0.010
lectin, galactose binding, soluble 3	1426808_at	6.14	6.19	6.97	0.83	0.015

solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4	1424562_a_at	6.68	6.84	7.51	0.83	0.012
glial cell line derived neurotrophic factor family receptor alpha 1	1450440_at	8.86	9.27	9.68	0.82	0.005
solute carrier organic anion transporter family, member 1a4	1420405_at	8.69	8.99	9.51	0.82	0.005
wee 1 homolog (S. pombe)	1416773_at	7.92	8.30	8.74	0.82	0.015
P450 (cytochrome) oxidoreductase	1416933_at	10.57	10.90	11.38	0.81	0.012
acyl-CoA synthetase long-chain family member 4	1451828_a_at	6.36	6.54	7.16	0.81	0.031
leptin receptor	1425875_a_at	4.57	4.58	5.38	0.81	0.013
disabled homolog 2 (Drosophila)	1420498_a_at	6.44	6.80	7.25	0.81	0.015
myristoylated alanine rich protein kinase C substrate	1415971_at	7.33	7.44	8.13	0.80	0.009
RIKEN cDNA D330050I23 gene	1434301_at	4.85	4.99	5.64	0.80	0.022
eosinophil-associated, ribonuclease A family, member 1 ///	1422411_s_at	4.42	4.38	5.21	0.79	0.013
eosinophil-associated, ribonuclease A family, member 2 ///						
eosinophil-associated, ribonuclease A family, member 3 ///						
eosinophil-associated, ribonuclease A family, member 12						
vanin 1	1447845_s_at	8.23	8.27	9.02	0.79	0.011
ATP-binding cassette, sub-family B (MDR/TAP), member 1A	1419759_at	3.99	3.86	4.79	0.79	0.032
endothelin receptor type B	1437347_at	6.47	6.84	7.26	0.79	0.030
methionine sulfoxide reductase B3	1454997_at	5.63	5.68	6.42	0.78	0.005
preimplantation protein 4	1429639_at	7.18	7.80	7.97	0.78	0.046
Kruppel-like factor 6	1447448_s_at	4.45	4.47	5.23	0.78	0.028
junction adhesion molecule 2	1449408_at	4.83	4.85	5.61	0.78	0.012
lymphocyte antigen 6 complex, locus A	1417185_at	8.21	8.52	8.99	0.78	0.019
cytotoxic T lymphocyte-associated protein 2 alpha	1448471_a_at	4.74	4.96	5.52	0.78	0.018
fibrinogen-like protein 1	1424599_at	10.95	11.12	11.73	0.78	0.006
ELK3, member of ETS oncogene family	1448797_at	5.69	5.88	6.47	0.77	0.026
epithelial membrane protein 2	1433670_at	6.67	6.90	7.44	0.77	0.013
heat shock protein 110	1423566_a_at	6.41	6.75	7.18	0.77	0.009
RIKEN cDNA 9330120H11 gene	1457671_at	6.32	6.78	7.09	0.77	0.019
leucine rich repeat containing 28	1433858_at	6.98	7.21	7.75	0.77	0.012
guanylate cyclase 1, soluble, alpha 3	1434141_at	5.54	5.38	6.31	0.77	0.043
RAB8B, member RAS oncogene family	1426799_at	5.67	6.00	6.44	0.77	0.015
RIKEN cDNA 4833408C14 gene	1420948_s_at	3.83	3.95	4.60	0.76	0.009
FYVE, RhoGEF and PH domain containing 5	1460578_at	5.47	5.71	6.23	0.76	0.040
transforming growth factor, beta receptor III	1433795_at	7.54	7.95	8.30	0.76	0.010

Protein tyrosine phosphatase, receptor type, B	1436367_at	8.68	8.84	9.44	0.76	0.010
myocyte enhancer factor 2A	1421252_a_at	5.43	5.65	6.19	0.75	0.010
solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member 3	1424580_at	6.99	7.12	7.74	0.75	0.026
protein tyrosine phosphatase 4a1	1419024_at	7.95	8.22	8.71	0.75	0.012
defensin beta 1	1419491_at	4.14	4.25	4.89	0.75	0.011
pyruvate kinase, muscle	1417308_at	5.70	5.77	6.45	0.75	0.016
DnaJ (Hsp40) homolog, subfamily C, member 12	1417441_at	8.54	8.88	9.29	0.75	0.005
prolactin receptor	1450226_at	8.38	8.44	9.13	0.75	0.008
CD9 antigen	1416066_at	8.23	8.37	8.97	0.75	0.009
acyl-CoA thioesterase 9	1418073_at	4.84	5.03	5.58	0.75	0.040
endothelial-specific receptor tyrosine kinase	1418788_at	5.79	5.94	6.54	0.75	0.013
EF hand domain containing 2	1431339_a_at	8.16	8.61	8.90	0.74	0.032
serum/glucocorticoid regulated kinase 3	1420918_at	6.18	6.45	6.91	0.74	0.031
homer homolog 2 (Drosophila)	1424367_a_at	8.26	8.51	8.99	0.74	0.037
vesicle-associated membrane protein 3	1421102_a_at	5.55	5.62	6.29	0.74	0.019
angiopoietin-like 2	1450085_at	4.67	4.96	5.40	0.73	0.046
expressed sequence AI314180	1456255_at	5.39	5.57	6.12	0.73	0.034
2,3-bisphosphoglycerate mutase	1415864_at	6.79	6.90	7.52	0.73	0.016
interferon-stimulated protein	1419569_a_at	5.60	5.55	6.33	0.73	0.014
amyloid beta (A4) precursor protein	1420621_a_at	7.35	7.54	8.07	0.72	0.010
tumor necrosis factor receptor superfamily, member 19	1425212_a_at	4.91	5.07	5.63	0.72	0.010
procollagen, type XIV, alpha 1	1427168_a_at	7.14	7.03	7.85	0.72	0.012
guanine nucleotide binding protein (G protein), gamma 11	1448942_at	7.09	7.17	7.80	0.71	0.014
protein tyrosine phosphatase, receptor type, G /// similar to protein tyrosine phosphatase, receptor type, G	1434360_s_at	6.07	6.20	6.79	0.71	0.035
CD55 antigen	1460242_at	5.15	5.22	5.86	0.71	0.019
moesin	1450379_at	5.26	5.30	5.97	0.71	0.031
CD163 antigen	1419144_at	6.58	6.64	7.29	0.71	0.012
interleukin 10 receptor, beta	1419455_at	6.82	6.88	7.53	0.70	0.011
prolactin receptor	1421382_at	8.50	8.49	9.20	0.70	0.010
Max interacting protein 1	1425732_a_at	6.68	6.91	7.39	0.70	0.018
RIKEN cDNA 6230424C14 gene	1441972_at	6.28	6.57	6.99	0.70	0.046
protein tyrosine phosphatase, receptor type, B	1427486_at	7.45	7.74	8.15	0.70	0.030
CD24a antigen	1448182_a_at	4.10	4.01	4.80	0.70	0.019
regulator of G-protein signaling 19	1417786_a_at	4.60	4.73	5.30	0.70	0.047
lumican	1423607_at	7.08	7.33	7.78	0.70	0.046

TCDD-inducible poly(ADP-ribose) polymerase	1452160_at	4.44	4.33	5.13	0.70	0.035
BCL2/adenovirus E1B interacting protein 3-like	1448525_a_at	7.93	8.02	8.62	0.69	0.020
cyclin D2	1434745_at	5.25	5.45	5.94	0.69	0.010
programmed cell death 6 interacting protein	1426184_a_at	6.69	6.91	7.38	0.69	0.021
Fc receptor, IgG, low affinity III	1448620_at	5.98	6.05	6.67	0.69	0.009
Cysteine and tyrosine-rich protein 1	1435701_at	6.37	6.58	7.06	0.69	0.022
Kruppel-like factor 13	1432543_a_at	7.49	8.03	8.18	0.69	0.015
solute carrier family 16 (monocarboxylic acid transporters), member 7	1448502_at	8.12	8.15	8.80	0.69	0.016
C-type lectin domain family 4, member n	1419627_s_at	4.90	5.13	5.58	0.68	0.035
ankyrin repeat domain 12	1435230_at	4.49	4.78	5.17	0.68	0.035
synaptotagmin I	1421990_at	5.28	5.67	5.96	0.68	0.009
glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	1421415_s_at	5.32	5.54	6.00	0.68	0.015
butyrylcholinesterase	1421218_at	6.92	6.80	7.60	0.68	0.017
RRN3 RNA polymerase I transcription factor homolog (yeast)	1415725_at	6.08	6.36	6.76	0.68	0.049
RAS, guanyl releasing protein 3	1438030_at	5.79	5.97	6.47	0.68	0.024
phosphoenolpyruvate carboxykinase 1, cytosolic	1439617_s_at	11.30	11.90	11.98	0.68	0.020
plasmalemma vesicle associated protein	1418090_at	7.97	7.93	8.65	0.68	0.019
RIKEN cDNA A230097K15 gene	1454799_at	4.85	4.81	5.53	0.68	0.032
annexin A1	1448213_at	5.88	5.61	6.55	0.68	0.024
ST3 beta-galactoside alpha-2,3-sialyltransferase 5	1449198_a_at	9.06	9.29	9.74	0.68	0.016
coiled-coil domain containing 80	1424186_at	6.60	6.64	7.27	0.67	0.009
STEAP family member 4	1425829_a_at	8.29	8.52	8.96	0.67	0.039
similar to Putative RNA-binding protein 3 (RNA-binding motif protein 3)	1422660_at	8.99	9.05	9.66	0.67	0.024
ubiquitination factor E4A, UFD2 homolog (S. cerevisiae)	1425384_a_at	6.77	7.11	7.43	0.67	0.030
cystin 1	1419070_at	5.65	5.96	6.31	0.67	0.010
interleukin 13 receptor, alpha 1	1427164_at	6.80	7.05	7.46	0.66	0.019
collectin sub-family member 10	1457871_at	7.49	7.54	8.15	0.66	0.032
S100 calcium binding protein A6 (calcyclin)	1421375_a_at	4.39	4.33	5.05	0.66	0.036
secreted acidic cysteine rich glycoprotein	1448392_at	8.03	8.02	8.69	0.66	0.015
superoxide dismutase 3, extracellular	1417633_at	7.24	7.36	7.89	0.66	0.012
microsomal glutathione S-transferase 3	1448300_at	8.13	8.02	8.78	0.66	0.003
growth differentiation factor 10	1424007_at	5.06	5.27	5.71	0.66	0.032
carbonyl reductase 1	1460196_at	9.80	9.76	10.45	0.66	0.009
G protein-coupled receptor 137B /// similar to	1429775_a_at	3.82	4.09	4.47	0.66	0.035

transmembrane 7 superfamily member 1						
glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	1451733_at	4.06	4.19	4.71	0.66	0.041
period homolog 3 (Drosophila)	1421087_at	5.20	5.62	5.86	0.66	0.045
protein kinase C, eta	1422079_at	3.84	4.10	4.49	0.65	0.033
heparanase	1433930_at	5.28	5.50	5.93	0.65	0.012
protein tyrosine phosphatase 4a2	1420612_s_at	7.90	7.98	8.55	0.65	0.022
DNA segment, Chr 5, ERATO Doi 593, expressed	1434442_at	10.08	10.36	10.73	0.65	0.043
endothelial cell-specific adhesion molecule	1460356_at	6.85	7.05	7.50	0.65	0.021
cytochrome P450, family 4, subfamily a, polypeptide 10 ///	1424853_s_at	11.20	11.29	11.85	0.65	0.019
cDNA sequence BC013476						
ERO1-like (S. cerevisiae)	1419030_at	6.53	6.88	7.18	0.65	0.040
Rap guanine nucleotide exchange factor (GEF) 5	1455840_at	4.19	4.34	4.84	0.64	0.015
mannose receptor, C type 1	1450430_at	8.01	8.22	8.65	0.64	0.033
bone morphogenetic protein 5	1421283_at	5.65	5.73	6.29	0.64	0.032
hemoglobin alpha, adult chain 1	1428361_x_at	13.02	13.13	13.66	0.64	0.019
armadillo repeat containing, X-linked 4	1427167_at	3.43	3.53	4.08	0.64	0.029
cytochrome b-245, beta polypeptide	1422978_at	4.86	5.09	5.50	0.64	0.043
hypothetical gene LOC433762	1431214_at	6.77	7.16	7.41	0.64	0.011
transient receptor potential cation channel, subfamily M, member 7	1416800_at	7.95	8.27	8.59	0.64	0.038
ras homolog gene family, member J	1418892_at	4.69	4.62	5.33	0.64	0.014
Down syndrome critical region homolog 1 (human)	1416601_a_at	5.76	5.96	6.39	0.63	0.045
glutathione S-transferase, mu 3	1427473_at	8.44	8.33	9.07	0.63	0.013
platelet-activating factor acetylhydrolase, isoform 1b, alpha2 subunit	1422793_at	6.40	6.71	7.03	0.63	0.026
tropomyosin 4	1433883_at	7.56	7.71	8.19	0.63	0.018
acyl-CoA thioesterase 4	1422077_at	4.20	4.39	4.83	0.63	0.015
myosin, light polypeptide 9, regulatory	1452670_at	5.06	4.80	5.69	0.63	0.005
2,3-bisphosphoglycerate mutase	1448119_at	7.60	7.72	8.23	0.63	0.016
solute carrier family 16 (monocarboxylic acid transporters), member 10	1434592_at	7.78	8.21	8.41	0.63	0.014
ADP-ribosylation factor-like 6 interacting protein 2	1431197_at	8.21	8.28	8.84	0.63	0.034
armadillo repeat containing, X-linked 3	1424373_at	6.85	6.96	7.48	0.63	0.017
triggering receptor expressed on myeloid cells-like 4	1460014_at	4.67	4.67	5.29	0.63	0.016
solute carrier family 40 (iron-regulated transporter), member 1	1448566_at	9.48	9.62	10.11	0.63	0.023
p300/CBP-associated factor	1450821_at	7.94	8.16	8.56	0.63	0.028

myristoylated alanine rich protein kinase C substrate	1415973_at	7.00	7.04	7.62	0.62	0.029
angiopoietin-like 2	1455090_at	6.93	7.26	7.56	0.62	0.009
protein phosphatase 1, regulatory (inhibitor) subunit 2	1417341_a_at	8.32	8.35	8.94	0.62	0.024
retinol dehydrogenase 9	1427963_s_at	10.52	10.78	11.14	0.62	0.022
Rho GTPase activating protein 5	1423194_at	6.16	6.22	6.79	0.62	0.009
avian musculoaponeurotic fibrosarcoma (v-maf) AS42	1447849_s_at	5.01	5.39	5.63	0.62	0.010
oncogene homolog						
EGF, latrophilin seven transmembrane domain containing 1	1418058_at	7.11	7.40	7.73	0.62	0.022
heterogeneous nuclear ribonucleoprotein A1	1423531_a_at	6.44	6.64	7.06	0.62	0.042
uridine phosphorylase 1	1448562_at	5.52	5.84	6.14	0.62	0.028
RIKEN cDNA 1500003O03 gene	1450007_at	9.73	9.95	10.35	0.62	0.025
predicted gene, OTTMUSG00000000971	1436530_at	9.33	9.32	9.95	0.62	0.016
procollagen C-endopeptidase enhancer protein	1437165_a_at	7.06	6.95	7.68	0.62	0.013
glutaredoxin	1416593_at	9.17	9.29	9.79	0.62	0.009
gap junction membrane channel protein alpha 1	1438945_x_at	3.83	3.99	4.45	0.62	0.048
transmembrane protein 47	1449885_at	3.70	3.60	4.31	0.62	0.038
protein phosphatase 1, regulatory (inhibitor) subunit 14B	1450914_at	7.92	8.11	8.54	0.61	0.011
forkhead box O1	1416981_at	7.01	7.34	7.62	0.61	0.046
ethanolamine kinase 1	1454633_at	6.42	6.48	7.03	0.61	0.028
---	1439794_at	6.44	6.41	7.06	0.61	0.030
tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	1452714_at	5.52	5.82	6.13	0.61	0.025
ubiquitin specific peptidase 34	1434393_at	5.71	5.94	6.32	0.61	0.018
erythrocyte protein band 4.1-like 5	1425199_a_at	8.52	8.74	9.13	0.61	0.018
myosin regulatory light chain interacting protein	1424988_at	7.59	7.96	8.20	0.61	0.019
chemokine (C-C motif) ligand 6	1417266_at	6.36	6.24	6.97	0.61	0.022
serine (or cysteine) peptidase inhibitor, clade B, member 6a	1450138_a_at	7.36	7.48	7.97	0.61	0.045
vascular endothelial growth factor B	1451803_a_at	6.91	6.82	7.51	0.61	0.024
aldo-keto reductase family 1, member B8	1448894_at	5.90	6.10	6.51	0.60	0.016
lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	1444487_at	6.81	6.73	7.41	0.60	0.048
endomucin	1425582_a_at	5.32	5.44	5.92	0.60	0.020
glypican 6	1428774_at	3.90	4.12	4.50	0.60	0.025
Transcribed locus	1444052_at	7.34	7.72	7.94	0.60	0.024
prolactin receptor	1425853_s_at	8.98	8.99	9.57	0.60	0.035
E26 avian leukemia oncogene 2, 3prime domain	1416268_at	7.07	7.29	7.67	0.59	0.018
four and a half LIM domains 1	1417872_at	6.76	6.67	7.35	0.59	0.019

baculoviral IAP repeat-containing 4	1456088_at	7.46	7.59	8.05	0.59	0.012
myosin IC	1419648_at	6.19	6.34	6.79	0.59	0.046
sorting nexin 10	1431055_a_at	7.93	8.11	8.52	0.59	0.032
muscleblind-like 2	1436858_at	7.56	7.75	8.15	0.59	0.022
hemoglobin, beta adult major chain /// hemoglobin, beta adult minor chain	1417184_s_at	12.58	12.62	13.17	0.59	0.015
solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4	1455069_x_at	5.93	5.93	6.52	0.59	0.010
protein tyrosine phosphatase 4a1 /// predicted gene, EG269859 /// protein tyrosine phosphatase 4a1-like /// similar to protein tyrosine phosphatase 4a1 /// protein tyrosine phosphatase 4a1 pseudogene /// predicted gene, EG667723	1449322_at	9.99	10.08	10.58	0.59	0.022
phosphoribosyl pyrophosphate amidotransferase	1452831_s_at	7.25	7.61	7.84	0.59	0.018
RIKEN cDNA 2900019G14 gene	1438194_at	8.90	9.24	9.49	0.59	0.017
gamma-glutamyl hydrolase /// similar to Gamma-glutamyl hydrolase precursor (Gamma-Glu-x carboxypeptidase) (Conjugase) (GH) (Folypolyglutamate hydrolase) (FGPH)	1419595_a_at	6.81	6.72	7.40	0.59	0.021
RIKEN cDNA 4931408D14 gene	1431806_at	7.03	7.33	7.61	0.59	0.049
spindlin 1	1415795_at	6.75	6.90	7.33	0.59	0.049
ATPase, Na+/K+ transporting, beta 1 polypeptide	1439036_a_at	7.91	7.84	8.50	0.59	0.033
S100 calcium binding protein A11 (calgizzarin)	1460351_at	6.82	6.92	7.41	0.58	0.022
phosphatidylinositol 3-kinase, C2 domain containing, alpha polypeptide	1425862_a_at	5.49	5.63	6.07	0.58	0.030
receptor (calcitonin) activity modifying protein 2	1418187_at	6.31	6.51	6.89	0.58	0.043
RIKEN cDNA 1110006O17 gene	1437451_at	4.91	4.76	5.49	0.58	0.010
topoisomerase (DNA) II beta	1448458_at	4.13	4.26	4.71	0.58	0.043
nucleoporin like 1	1424389_at	5.75	5.89	6.32	0.58	0.031
retinoblastoma binding protein 4	1454875_a_at	8.95	9.16	9.52	0.58	0.034
tubulointerstitial nephritis antigen-like	1417109_at	7.54	7.85	8.12	0.58	0.019
multimerin 2	1456768_a_at	6.68	6.83	7.26	0.57	0.015
EGF-like domain 7	1451428_x_at	7.42	7.56	7.99	0.57	0.018
myosin IC	1449551_at	6.23	6.52	6.80	0.57	0.031
trans-acting transcription factor 1	1448994_at	4.35	4.56	4.92	0.57	0.029
platelet derived growth factor, alpha	1418711_at	5.94	5.94	6.51	0.57	0.031
cytochrome P450, family 2, subfamily c, polypeptide 38	1452501_at	8.71	8.50	9.28	0.57	0.012
preimplantation protein 4	1429144_at	10.61	11.24	11.18	0.57	0.028

sorting nexin 6	1451602_at	6.56	6.75	7.13	0.57	0.049
6 days neonate spleen cDNA, RIKEN full-length enriched library, clone:F430010L17 product:hypothetical protein, full insert sequence	1436357_at	5.33	5.41	5.90	0.57	0.035
cathepsin B	1417491_at	6.80	7.09	7.36	0.56	0.022
serine (or cysteine) peptidase inhibitor, clade A, member 6	1448506_at	11.51	11.65	12.07	0.56	0.019
peripheral myelin protein	1417133_at	5.86	5.75	6.42	0.56	0.022
metastasis suppressor 1	1424826_s_at	9.28	9.55	9.84	0.56	0.040
CD48 antigen	1427301_at	4.14	4.18	4.69	0.56	0.011
glycoprotein 49 A /// leukocyte immunoglobulin-like receptor, subfamily B, member 4	1420394_s_at	5.62	5.69	6.18	0.56	0.023
interleukin 6 signal transducer	1460295_s_at	6.72	7.05	7.28	0.56	0.034
nuclear distribution gene E-like homolog 1 (A. nidulans)	1422568_at	8.63	8.94	9.18	0.56	0.020
zinc finger CCCH type containing 7 A	1419898_s_at	5.08	5.05	5.64	0.56	0.037
KRR1, small subunit (SSU) processome component, homolog (yeast)	1452658_at	5.19	5.47	5.74	0.55	0.032
5prime-3prime exoribonuclease 2	1422842_at	7.16	7.43	7.71	0.55	0.035
UV radiation resistance associated gene	1454706_at	8.26	8.50	8.81	0.55	0.029
eosinophil-associated, ribonuclease A family, member 2	1449846_at	6.26	6.41	6.81	0.55	0.015
desmoplakin	1427610_at	4.99	5.29	5.54	0.55	0.044
TYRO protein tyrosine kinase binding protein	1450792_at	7.99	8.03	8.54	0.55	0.040
steroid 5 alpha-reductase 2-like	1456691_s_at	6.16	5.98	6.71	0.55	0.019
arsenic (+3 oxidation state) methyltransferase	1431980_a_at	7.97	7.86	8.52	0.55	0.013
RIKEN cDNA 2610027C15 gene	1454933_at	6.69	6.70	7.24	0.55	0.013
SH3 domain protein D19	1427574_s_at	5.51	5.66	6.05	0.54	0.037
cartilage associated protein	1448592_at	6.17	6.10	6.72	0.54	0.012
caveolin, caveolae protein 1	1449145_a_at	5.04	5.29	5.58	0.54	0.036
BRCA1 associated protein	1416739_a_at	7.53	7.78	8.07	0.54	0.043
MAM domain containing 2	1453152_at	4.91	5.00	5.45	0.54	0.015
ATP-binding cassette, sub-family B (MDR/TAP), member 1A	1419758_at	3.58	3.59	4.12	0.54	0.039
expressed sequence A1117581	1436233_at	5.68	6.09	6.22	0.54	0.022
potassium inwardly-rectifying channel, subfamily J, member 8	1418142_at	6.11	6.19	6.65	0.54	0.023
insulin-like growth factor binding protein 3	1458268_s_at	6.85	6.58	7.39	0.54	0.029
hemoglobin alpha, adult chain 1	1452757_s_at	12.65	12.73	13.19	0.54	0.020
Ngfi-A binding protein 1	1448781_at	7.78	7.87	8.31	0.54	0.024

sorting nexin 13	1454938_at	7.25	7.34	7.78	0.54	0.026
G protein-coupled receptor 177	1423824_at	4.45	4.68	4.98	0.53	0.032
inhibitor of DNA binding 4	1423259_at	4.26	4.52	4.79	0.53	0.031
phosphodiesterase 2A, cGMP-stimulated	1452202_at	6.23	6.36	6.76	0.53	0.033
poly (A) polymerase alpha	1455836_at	7.61	7.67	8.13	0.53	0.027
coiled-coil domain containing 101	1451167_at	6.47	6.43	6.99	0.53	0.034
vanin 1	1418486_at	10.07	10.26	10.60	0.53	0.016
CD36 antigen	1450883_a_at	8.38	7.77	8.91	0.53	0.009
cysteine and glycine-rich protein 1	1425811_a_at	5.51	5.32	6.03	0.52	0.009
solute carrier family 35 (UDP-N-acetylglucosamine (UDP-GlcNAc) transporter), member 3	1424579_at	7.29	7.51	7.82	0.52	0.015
glial cell line derived neurotrophic factor family receptor alpha 1	1439015_at	9.41	9.55	9.93	0.52	0.036
serine/threonine kinase 3 (Ste20, yeast homolog)	1418513_at	5.31	5.51	5.83	0.52	0.034
CD36 antigen	1423166_at	8.58	8.23	9.10	0.52	0.028
G protein-coupled receptor 116	1440830_at	7.26	7.54	7.79	0.52	0.022
serine (or cysteine) peptidase inhibitor, clade H, member 1	1450843_a_at	5.36	5.38	5.88	0.52	0.028
thymosin, beta 10	1436902_x_at	7.26	7.07	7.78	0.52	0.022
EF hand domain containing 2	1437478_s_at	10.41	10.85	10.93	0.52	0.042
Tax1 (human T-cell leukemia virus type I) binding protein 3 /// ribosomal protein L13	1455871_s_at	8.19	8.21	8.70	0.52	0.043
attractin	1421166_at	6.09	6.23	6.61	0.52	0.032
retinitis pigmentosa 2 homolog (human)	1419585_at	4.45	4.50	4.96	0.52	0.022
stromal antigen 2	1450396_at	7.62	7.82	8.14	0.52	0.027
matrix metalloproteinase 14 (membrane-inserted)	1448383_at	7.51	7.71	8.03	0.52	0.021
transmembrane protein 106B	1452290_at	8.53	8.56	9.04	0.51	0.048
forkhead box K2	1452753_at	6.67	6.77	7.18	0.51	0.042
sorting nexin 2	1460224_at	9.26	9.37	9.77	0.51	0.035
pyruvate dehydrogenase kinase, isoenzyme 1	1423748_at	8.38	8.32	8.89	0.51	0.037
arrestin, beta 1	1460444_at	5.15	5.07	5.65	0.51	0.024
phosphatidylinositol 4-kinase type 2 beta	1449862_a_at	7.13	7.29	7.64	0.51	0.022
cyclin-dependent kinase 4 /// similar to Cell division protein kinase 4 (Cyclin-dependent kinase 4) (PSK-J3) (CRK3)	1422440_at	7.33	7.40	7.84	0.51	0.035
structural maintenance of chromosomes 6	1417736_at	5.07	5.30	5.57	0.51	0.046
eukaryotic translation termination factor 1	1451208_at	8.30	8.49	8.80	0.51	0.035
Rho, GDP dissociation inhibitor (GDI) beta	1426454_at	7.05	7.12	7.56	0.50	0.017
testis-specific protein, Y-encoded-like 1	1460717_at	6.21	6.33	6.72	0.50	0.042

tyrosine kinase receptor 1	1416238_at	5.55	5.59	6.06	0.50	0.026
amyloid beta (A4) precursor protein	1427442_a_at	9.32	9.50	9.82	0.50	0.023
serine (or cysteine) peptidase inhibitor, clade A, member 3K	1423867_at	8.16	8.62	8.66	0.50	0.028
glutaredoxin	1416592_at	8.94	9.00	9.44	0.50	0.022
septin 4	1448729_a_at	6.52	6.28	7.01	0.50	0.009
vacuolar protein sorting 26 homolog A (yeast)	1420495_a_at	8.59	8.74	9.09	0.50	0.020
dehydrogenase/reductase (SDR family) member 7	1426440_at	8.52	8.58	9.02	0.49	0.017
aldolase 1, A isoform /// predicted gene, EG667483 ///	1439375_x_at	8.43	8.53	8.92	0.49	0.041
similar to Fructose-bisphosphate aldolase A (Muscle-type aldolase) (Aldolase 1)						
C-type lectin domain family 1, member b	1421182_at	7.93	8.03	8.42	0.49	0.049
phosphatidylinositol transfer protein, cytoplasmic 1	1435066_at	6.81	6.98	7.31	0.49	0.044
---	1432176_a_at	6.77	6.90	7.27	0.49	0.050
stromal antigen 1	1421940_at	5.09	5.09	5.58	0.49	0.013
membrane interacting protein of RGS16	1418444_a_at	9.52	9.66	10.01	0.49	0.035
myeloid ecotropic viral integration site-related gene 1	1457632_s_at	4.07	4.46	4.56	0.49	0.028
RIKEN cDNA 9030411M13 gene	1431371_at	3.48	3.49	3.96	0.49	0.047
protein kinase N2	1437296_at	6.47	6.66	6.96	0.48	0.033
transforming, acidic coiled-coil containing protein 1	1429591_at	5.08	5.18	5.56	0.48	0.042
RIKEN cDNA A230067G21 gene	1455750_at	5.97	6.08	6.46	0.48	0.028
aldolase 1, A isoform	1434799_x_at	8.77	8.82	9.25	0.48	0.024
RIKEN cDNA 1810058I24 gene	1436339_at	10.70	10.90	11.18	0.48	0.033
leucine rich repeat containing G protein coupled receptor 5	1450988_at	5.43	5.71	5.91	0.48	0.043
interleukin 6 signal transducer	1452843_at	8.69	8.91	9.17	0.48	0.035
expressed sequence AW146242	1451127_at	4.29	4.22	4.77	0.48	0.048
BCL2/adenovirus E1B interacting protein 1, NIP3	1422470_at	11.47	11.59	11.95	0.48	0.031
transmembrane 6 superfamily member 2	1425079_at	8.41	8.39	8.89	0.48	0.022
nuclear prelamin A recognition factor	1451678_at	8.95	9.41	9.43	0.47	0.035
RIKEN cDNA 4932442K08 gene	1417142_at	7.19	7.44	7.66	0.47	0.023
CAP-GLY domain containing linker protein 1	1431098_at	3.55	3.69	4.02	0.47	0.048
reticulon 4	1421116_a_at	9.02	9.00	9.49	0.47	0.027
pyruvate dehydrogenase E1 alpha 1	1449137_at	9.72	9.81	10.18	0.46	0.040
ATP-binding cassette, sub-family G (WHITE), member 5	1419393_at	9.74	10.16	10.20	0.46	0.033
RIKEN cDNA 2210013O21 gene	1430538_at	5.07	5.08	5.53	0.46	0.024
palmelphin	1417251_at	8.63	8.65	9.09	0.46	0.045
TLC domain containing 1	1426616_at	5.99	5.93	6.45	0.46	0.036
solute carrier family 16 (monocarboxylic acid transporters),	1436368_at	8.00	8.26	8.46	0.46	0.043

member 10							
synaptosomal-associated protein	1423355_at	6.23	6.21	6.68	0.46	0.022	
NAD kinase	1416249_at	9.54	9.72	9.99	0.45	0.029	
RIKEN cDNA A530057A03 gene	1456208_at	4.33	4.20	4.78	0.45	0.043	
acetyl-Coenzyme A carboxylase alpha // similar to acetyl-coenzyme A carboxylase alpha	1427595_at	4.72	4.67	5.17	0.45	0.045	
transmembrane 4 superfamily member 4	1424962_at	10.01	10.16	10.46	0.45	0.036	
caspase 1	1449265_at	4.76	4.73	5.21	0.45	0.027	
ubiquitin specific peptidase 1	1423675_at	6.08	5.87	6.52	0.45	0.032	
procollagen, type I, alpha 2	1423110_at	4.05	4.20	4.50	0.45	0.045	
phosphatidylinositol transfer protein, cytoplasmic 1	1455204_at	6.50	6.51	6.94	0.45	0.032	
splicing factor, arginine/serine-rich 2 (SC-35)	1452439_s_at	8.44	8.42	8.88	0.44	0.022	
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	1420828_s_at	9.82	9.93	10.26	0.44	0.028	
activation protein, theta polypeptide							
RIKEN cDNA E030018N11 gene	1435777_at	6.15	6.09	6.59	0.44	0.049	
RIKEN cDNA 4631422O05 gene	1428861_at	4.57	4.41	5.01	0.44	0.017	
Btg3 associated nuclear protein	1452462_a_at	5.92	5.83	6.36	0.44	0.033	
legumain	1448883_at	7.54	7.57	7.97	0.44	0.044	
SLAIN motif family, member 2	1427024_at	4.46	4.50	4.89	0.44	0.045	
RIKEN cDNA 1700052N19 gene	1421495_a_at	5.70	5.86	6.14	0.43	0.036	
Son cell proliferation protein	1420951_a_at	6.77	7.04	7.20	0.43	0.039	
activating transcription factor 6	1456021_at	6.72	6.92	7.16	0.43	0.028	
procollagen C-endopeptidase enhancer protein	1448433_a_at	5.76	5.62	6.18	0.43	0.040	
procollagen, type IV, alpha 3 (Goodpasture antigen) binding protein	1449847_a_at	4.15	4.29	4.57	0.43	0.029	
activating transcription factor 6	1453288_at	6.31	6.43	6.73	0.42	0.049	
decorin	1449368_at	10.19	10.09	10.60	0.42	0.032	
cadherin 5	1433956_at	5.60	5.58	6.02	0.42	0.035	
angiomin-like 1	1455247_at	4.57	4.62	4.99	0.42	0.028	
dynammin 1-like	1452638_s_at	5.10	5.23	5.52	0.42	0.037	
ATP-binding cassette, sub-family C (CFTR/MRP), member 9	1435751_at	6.92	6.96	7.33	0.41	0.040	
extracellular matrix protein 1	1448613_at	9.59	9.62	10.00	0.41	0.042	
cysteine and histidine-rich domain (CHORD)-containing, zinc-binding protein 1	1460645_at	7.82	8.01	8.22	0.40	0.041	
taurine upregulated gene 1	1456398_at	6.54	6.53	6.94	0.39	0.029	
receptor (TNFRSF)-interacting serine-threonine kinase 1	1419508_at	6.37	6.30	6.76	0.39	0.040	

mannoside acetylglucosaminyltransferase 2	1452037_at	8.67	8.79	9.05	0.38	0.043
RIKEN cDNA B230219D22 gene	1424005_at	8.85	8.87	9.22	0.37	0.048
NTF2-related export protein 1	1422488_at	7.29	7.25	7.65	0.37	0.046
thymosin, beta 10	1437185_s_at	9.37	9.31	9.73	0.37	0.035
thioesterase superfamily member 2	1417316_at	9.93	9.91	10.29	0.36	0.047
adaptor-related protein complex AP-4, sigma 1	1451665_a_at	8.76	8.76	9.12	0.36	0.047
frizzled homolog 4 (Drosophila)	1449416_at	3.23	3.05	3.58	0.35	0.042
N-acetylneuramate pyruvate lyase	1424265_at	4.97	4.78	5.31	0.34	0.039
RIKEN cDNA 4432416J03 gene	1453220_at	7.16	7.10	7.46	0.30	0.049
cyclin D1	1417420_at	6.98	7.61	6.86	-0.12	0.034
retinoic acid receptor responder (tazarotene induced) 1	1438055_at	10.33	10.48	9.98	-0.35	0.022
RIKEN cDNA 1700071A11 gene	1439967_at	5.12	5.12	4.76	-0.36	0.048
RIKEN cDNA 0610012H03 gene	1453043_at	8.68	8.70	8.32	-0.36	0.041
cDNA sequence BC026585	1460696_at	8.04	8.04	7.68	-0.36	0.049
hydroxysteroid (17-beta) dehydrogenase 2	1418352_at	11.52	11.56	11.15	-0.37	0.046
dpy-19-like 3 (C. elegans)	1437858_at	5.20	5.57	4.82	-0.38	0.029
1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta)	1428821_at	11.35	11.11	10.96	-0.39	0.048
inositol 1,3,4-triphosphate 5/6 kinase	1426733_at	8.22	7.97	7.83	-0.39	0.039
lon peptidase 2, peroxisomal	1460178_at	11.34	11.28	10.94	-0.40	0.029
isovaleryl coenzyme A dehydrogenase	1449001_at	9.72	9.69	9.33	-0.40	0.035
threonyl-tRNA synthetase	1460323_at	9.63	9.24	9.23	-0.40	0.022
polymerase (DNA directed), beta	1434230_at	8.33	8.24	7.93	-0.40	0.039
Mus musculus, clone IMAGE:4222153, mRNA	1439849_at	4.60	4.41	4.19	-0.41	0.043
aldehyde dehydrogenase 1 family, member L1	1424400_a_at	12.77	12.65	12.35	-0.41	0.036
glucosamine-6-phosphate deaminase 1 /// similar to Glucosamine-6-phosphate isomerase (Glucosamine-6- phosphate deaminase) (GNPDA) (GlcN6P deaminase) (Oscillin) /// predicted gene, EG667410	1448163_at	8.34	8.45	7.93	-0.42	0.036
ATP-binding cassette, sub-family G (WHITE), member 2	1422906_at	10.10	10.10	9.68	-0.42	0.020
adrenergic receptor, alpha 1b	1422183_a_at	7.46	7.19	7.04	-0.42	0.043
sarcosine dehydrogenase	1448426_at	10.19	10.14	9.75	-0.44	0.028
ATP-binding cassette, sub-family B (MDR/TAP), member 11	1449817_at	10.97	10.97	10.53	-0.44	0.020
RIKEN cDNA 2310045N14 gene	1454547_at	4.72	4.53	4.28	-0.44	0.047
MIF4G domain containing	1451523_a_at	8.80	8.65	8.36	-0.44	0.047
ATP citrate lyase	1451666_at	10.82	10.43	10.37	-0.45	0.039

protein kinase, cAMP dependent, catalytic, alpha	1450519_a_at	8.44	8.21	7.98	-0.46	0.043
acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	1434555_at	9.04	8.71	8.58	-0.46	0.023
orphan short chain dehydrogenase/reductase	1430785_at	8.58	8.58	8.11	-0.47	0.022
kelch-like 22 (Drosophila)	1426481_at	7.63	7.38	7.15	-0.47	0.050
dehydrogenase/reductase (SDR family) member 3	1448390_a_at	10.04	9.81	9.56	-0.47	0.022
complement component 9	1422815_at	12.58	12.58	12.10	-0.47	0.018
cytochrome P450, family 4, subfamily v, polypeptide 3	1417071_s_at	12.13	12.15	11.66	-0.47	0.012
amidohydrolase domain containing 1	1427370_at	11.16	11.06	10.69	-0.48	0.028
RIKEN cDNA 1300002K09 gene	1453234_at	10.01	9.99	9.53	-0.48	0.022
solute carrier family 25 (mitochondrial carrier, glutamate), member 22	1452653_at	9.13	9.20	8.65	-0.48	0.015
RIKEN cDNA 2310002J21 gene	1456393_at	11.48	11.21	11.00	-0.48	0.029
calyntenin 3	1426989_at	7.97	7.72	7.49	-0.49	0.018
apolipoprotein L 3 /// RIKEN cDNA 2210421G13 gene ///	1429272_a_at	10.01	9.90	9.52	-0.49	0.040
apolipoprotein L, 3-like /// similar to apolipoprotein L, 3 (predicted)						
---	1432732_at	4.69	4.52	4.20	-0.49	0.049
DNA-damage inducible protein 2	1429094_at	8.82	8.73	8.32	-0.50	0.029
endothelial cell growth factor 1 (platelet-derived)	1432181_s_at	9.93	9.82	9.43	-0.50	0.023
RIKEN cDNA 4930504E06 gene	1438321_x_at	10.37	10.16	9.87	-0.51	0.040
solute carrier family 30, member 10	1439934_at	7.60	7.24	7.09	-0.51	0.036
RIKEN cDNA 1110067D22 gene	1424318_at	7.80	7.33	7.29	-0.51	0.031
methylthioadenosine phosphorylase	1424425_a_at	8.86	8.61	8.34	-0.52	0.033
lectin, galactose binding, soluble 1	1455439_a_at	9.45	8.93	8.93	-0.52	0.043
histone cluster 1, H4h /// histone cluster 1, H4c /// histone cluster 1, H4i /// histone cluster 1, H4j /// histone cluster 1, H4k /// histone cluster 1, H4m /// histone cluster 1, H4a /// histone cluster 1, H4b	1422948_s_at	7.80	7.74	7.27	-0.52	0.049
tubulin, alpha 1C /// predicted gene, EG626534	1448232_x_at	7.16	7.05	6.64	-0.53	0.048
RIKEN cDNA 1500005A01 gene	1418474_at	8.71	8.69	8.17	-0.54	0.022
LIM domain containing 2 /// similar to epithelial protein lost in neoplasm	1456377_x_at	9.50	9.38	8.96	-0.54	0.034
tissue factor pathway inhibitor 2	1418547_at	10.59	10.36	10.04	-0.55	0.049
Rho GTPase activating protein 26	1444128_at	7.48	7.64	6.92	-0.56	0.018
---	1452433_at	4.92	4.62	4.34	-0.59	0.028
cytochrome P450, family 4, subfamily a, polypeptide 12	1424352_at	12.00	11.88	11.40	-0.59	0.047

EPS8-like 2	1417843_s_at	8.47	8.35	7.87	-0.60	0.022
cytochrome P450, family 4, subfamily v, polypeptide 3	1417070_at	11.17	11.13	10.56	-0.61	0.025
cytochrome P450, family 8, subfamily b, polypeptide 1	1449309_at	11.85	11.63	11.24	-0.61	0.022
folylpolyglutamyl synthetase	1460673_at	9.49	9.12	8.87	-0.62	0.011
cytochrome P450, family 2, subfamily u, polypeptide 1	1453500_at	8.19	8.35	7.57	-0.62	0.003
molybdenum cofactor sulfurase	1429352_at	9.47	9.20	8.85	-0.63	0.009
guanylate nucleotide binding protein 1	1420549_at	4.80	4.76	4.16	-0.64	0.021
melanocortin 2 receptor accessory protein	1451371_at	9.36	8.97	8.72	-0.65	0.013
immunity-related GTPase family, M	1418825_at	8.90	8.59	8.25	-0.65	0.029
histidine ammonia lyase /// similar to Histidine ammonia-lyase (Histidase)	1418645_at	11.25	11.08	10.60	-0.65	0.023
interferon-induced protein with tetratricopeptide repeats 1	1450783_at	6.08	5.75	5.42	-0.66	0.018
lipocalin 13	1428022_at	5.38	5.89	4.72	-0.66	0.035
---	1442537_at	9.70	9.91	9.04	-0.66	0.019
presenilin 2	1425869_a_at	11.38	11.12	10.70	-0.68	0.009
ATPase, class I, type 8B, member 4	1439814_at	6.14	6.07	5.45	-0.68	0.017
deltex 4 homolog (Drosophila)	1455711_at	6.33	6.10	5.64	-0.69	0.022
synaptic nuclear envelope 1	1455493_at	6.57	6.38	5.89	-0.69	0.021
yippee-like 2 (Drosophila)	1434277_a_at	7.71	7.38	7.02	-0.69	0.045
acyl-CoA synthetase short-chain family member 2	1422479_at	11.16	10.66	10.46	-0.70	0.030
circadian locomotor output cycles kaput /// hypothetical protein LOC620729	1418660_at	6.24	5.97	5.54	-0.70	0.038
serum/glucocorticoid regulated kinase 2	1418739_at	10.45	10.23	9.75	-0.70	0.005
sulfotransferase family 5A, member 1	1449816_at	8.29	8.59	7.59	-0.70	0.009
claudin 1	1437932_a_at	9.35	8.99	8.65	-0.70	0.024
predicted gene, ENSMUSG00000073019	1440522_at	4.44	4.36	3.74	-0.70	0.012
deltex 4 homolog (Drosophila)	1436545_at	6.76	6.57	6.04	-0.72	0.009
circadian locomotor output cycles kaput	1435775_at	6.72	6.53	6.00	-0.72	0.045
hypothetical protein LOC666185 /// hypothetical protein LOC666222 /// hypothetical protein LOC673030	1455240_x_at	7.37	6.61	6.64	-0.72	0.029
2prime-5prime oligoadenylate synthetase-like 1	1424339_at	6.80	6.56	6.07	-0.73	0.010
sushi domain containing 4	1460593_at	8.64	8.54	7.91	-0.73	0.005
leucine rich repeat and fibronectin type III domain containing 3	1456767_at	6.23	5.73	5.50	-0.73	0.025
RIKEN cDNA 5730414N17 gene	1429899_at	7.62	7.38	6.88	-0.74	0.017
dimethylarginine dimethylaminohydrolase 1	1429298_at	10.29	10.21	9.54	-0.75	0.017
suppression of tumorigenicity 5	1428372_at	7.47	7.00	6.72	-0.75	0.035

dimethylarginine dimethylaminohydrolase 1	1454995_at	9.27	9.16	8.50	-0.77	0.014
caseinolytic peptidase X (E.coli)	1423447_at	9.80	9.29	9.02	-0.78	0.049
dimethylarginine dimethylaminohydrolase 1	1455400_at	8.92	8.67	8.12	-0.80	0.013
solute carrier family 30, member 10	1438751_at	6.46	6.15	5.66	-0.80	0.022
---	1442051_at	5.81	5.75	5.00	-0.80	0.019
ethylmalonic encephalopathy 1	1417203_at	10.95	10.65	10.15	-0.80	0.004
insulin induced gene 2	1417982_at	11.08	10.81	10.27	-0.80	0.003
glycine decarboxylase	1416049_at	9.76	9.48	8.94	-0.82	0.004
7-dehydrocholesterol reductase	1448619_at	9.62	9.16	8.80	-0.82	0.007
choline kinase alpha	1450264_a_at	6.21	5.51	5.39	-0.83	0.043
dopachrome tautomerase	1418028_at	8.65	8.34	7.80	-0.85	0.010
camello-like 5	1424811_at	8.56	8.18	7.71	-0.85	0.014
tripartite motif protein 2	1417027_at	7.47	7.09	6.61	-0.87	0.032
chaperone, ABC1 activity of bc1 complex like (S. pombe)	1417066_at	10.20	9.77	9.32	-0.87	0.003
acid phosphatase, prostate	1441975_at	6.41	5.93	5.52	-0.89	0.015
insulin induced gene 2	1417981_at	9.90	9.42	9.00	-0.90	0.009
melanoregulin	1437250_at	10.05	9.68	9.13	-0.92	0.014
camello-like 4	1419520_at	9.17	9.08	8.25	-0.92	0.002
RIKEN cDNA 3010026O09 gene	1448930_at	6.63	5.98	5.71	-0.92	0.030
serum amyloid A 1	1450788_at	10.66	10.70	9.74	-0.92	0.010
chaperone, ABC1 activity of bc1 complex like (S. pombe)	1417067_s_at	8.03	7.66	7.11	-0.92	0.003
protein phosphatase 1, regulatory (inhibitor) subunit 3C	1425631_at	8.71	8.65	7.77	-0.94	0.044
procollagen, type V, alpha 3	1419703_at	6.56	6.04	5.62	-0.94	0.015
carbonic anhydrase 14	1450725_s_at	9.14	8.87	8.19	-0.95	0.003
dopa decarboxylase	1426215_at	10.13	9.66	9.18	-0.95	0.012
paroxysmal nonkinesigenic dyskinesia	1418746_at	9.76	9.42	8.79	-0.97	0.009
carboxylesterase 2 /// similar to carboxylesterase 2	1424245_at	11.34	11.40	10.32	-1.02	0.001
cDNA sequence BC015286	1457619_at	6.08	6.01	5.03	-1.04	0.011
aquaporin 8	1417828_at	9.66	9.05	8.56	-1.10	0.001
protein phosphatase 1, regulatory (inhibitor) subunit 3C	1433691_at	10.12	9.93	9.02	-1.10	0.013
RIKEN cDNA 1810046K07 gene	1453547_at	6.32	5.71	5.21	-1.10	0.003
RIKEN cDNA 2310043N10 gene	1428083_at	8.51	7.75	7.40	-1.11	0.031
early growth response 1	1417065_at	7.13	6.89	5.91	-1.22	0.011
UDP glucuronosyltransferase 2 family, polypeptide B38	1423397_at	8.94	9.03	7.69	-1.25	0.011
elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	1420722_at	11.42	11.15	10.15	-1.27	0.002
oligodendrocyte transcription factor 1	1416149_at	7.10	6.87	5.83	-1.28	0.007

macrophage activation 2 like	1447927_at	6.67	6.32	5.23	-1.44	0.003
macrophage activation 2 like /// macrophage activation 2 like LOC626578 /// similar to macrophage activation 2 like	1438676_at	7.43	6.93	5.97	-1.46	0.005
tubulin, beta 2a /// tubulin, beta 2b /// predicted gene, EG665524 /// similar to tubulin, beta 3	1427347_s_at	9.62	8.48	8.05	-1.57	0.009
immunoglobulin heavy chain 6 (heavy chain of IgM)	1427329_a_at	7.87	7.00	6.29	-1.58	0.030
immunoglobulin heavy chain 6 (heavy chain of IgM)	1427351_s_at	8.83	7.97	7.24	-1.60	0.028

## CHAPTER 2: SUPPLEMENTAL TABLES

Table 1: 121 probesets showing differential gene expression in high altitude versus low altitude mice.

Gene Title	Affymetrix Probeset ID	One way Adj pval	Nested pval Altitude	Fold Change
<b>Significant differential expression in both Two way and Nested ANOVA analyses</b>				
---	1457267_at	0.029	0.0050	-1.31
5, 10-methenyltetrahydrofolate synthetase	1460257_a_at	0.039	0.0071	1.56
aldehyde dehydrogenase 16 family, member A1	1447372_at	0.011	0.0027	-1.65
<b>annexin A11</b>	<b>1418468_at</b>	<b>0.031</b>	<b>0.0056</b>	<b>2.39</b>
ATPase, class V, type 10A	1447851_x_at	0.047	0.0096	-1.47
baculoviral IAP repeat-containing 4	1437533_at	0.003	0.0014	-1.35
CASP8 and FADD-like apoptosis regulator	1425686_at	0.040	0.0077	-1.68
cDNA sequence BC038822	1436608_at	0.039	0.0071	1.20
cDNA sequence BC048355	1460713_at	0.029	0.0065	-1.38
chemokine (C-C motif) ligand 19	1449277_at	0.040	0.0075	1.51
CHMP family, member 7	1451300_a_at	0.028	0.0047	1.26
<b>cytidine deaminase</b>	<b>1427357_at</b>	<b>0.006</b>	<b>0.0052</b>	<b>3.02</b>
cytochrome P450, family 2, subfamily c, polypeptide 39	1421363_at	0.029	0.0086	-1.59
cytoplasmic FMR1 interacting protein 2	1428347_at	0.036	0.0064	1.39
desmocollin 2	1421156_a_at	0.040	0.0086	1.34
dual specificity phosphatase 2	1450698_at	0.037	0.0093	1.26
<b>dynein light chain Tctex-type 1</b>	<b>1428116_a_at</b>	<b>0.015</b>	<b>0.0037</b>	<b>-2.99</b>
<b>dynein light chain Tctex-type 2</b>	<b>1453473_a_at</b>	<b>0.020</b>	<b>0.0039</b>	<b>-3.36</b>
dystroglycan 1	1426778_at	0.019	0.0037	1.18
E74-like factor 3	1416916_at	0.029	0.0088	1.17
<b>EGL nine homolog 3 (C. elegans)</b>	<b>1418649_at</b>	<b>0.019</b>	<b>0.0059</b>	<b>-2.10</b>
eukaryotic translation initiation factor 3, subunit I	1416233_at	0.040	0.0075	-1.10
expressed sequence AA536717	1435686_at	0.019	0.0096	-1.42
<b>expressed sequence AI450326</b>	<b>1442124_at</b>	<b>0.011</b>	<b>0.0027</b>	<b>2.51</b>

hypothetical protein C730026J16	1437650_at	0.036	0.0065	-1.26
immunoglobulin heavy chain 2 (serum IgA)	1429381_x_at	0.040	0.0078	1.40
<b>Immunoglobulin heavy chain 6 (heavy chain of IgM)</b>	<b>1425247_a_at</b>	<b>0.029</b>	<b>0.0052</b>	<b>2.49</b>
<b>Immunoglobulin heavy chain 6 (heavy chain of IgM)</b>	<b>1425324_x_at</b>	<b>0.029</b>	<b>0.0053</b>	<b>3.18</b>
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1427870_x_at	0.034	0.0060	1.89
<b>immunoglobulin joining chain</b>	<b>1424305_at</b>	<b>0.031</b>	<b>0.0057</b>	<b>5.26</b>
<b>immunoglobulin kappa chain, constant region</b>	<b>1427455_x_at</b>	<b>0.033</b>	<b>0.0059</b>	<b>5.90</b>
<b>immunoglobulin kappa chain, constant region</b>	<b>1427660_x_at</b>	<b>0.040</b>	<b>0.0075</b>	<b>4.63</b>
LMBR1 domain containing 1	1425186_at	0.021	0.0063	1.25
membrane-associated ring finger (C3HC4) 7	1440966_at	0.040	0.0079	-1.29
methythioadenosine phosphorylase	1424425_a_at	0.019	0.0037	1.57
<b>mitochondrial ribosomal protein L15</b>	<b>1430798_x_at</b>	<b>0.040</b>	<b>0.0076</b>	<b>-4.04</b>
mitogen-activated protein kinase-activated protein kinase 3	1434815_a_at	0.028	0.0085	1.57
motile sperm domain containing 3	1460452_at	0.020	0.0048	-1.21
neuroblastoma ras oncogene	1422687_at	0.019	0.0035	1.72
<b>NIPA-like domain containing 1</b>	<b>1453345_at</b>	<b>0.040</b>	<b>0.0079</b>	<b>4.02</b>
Notch gene homolog 1 (Drosophila)	1418634_at	0.034	0.0061	1.34
PDGFA associated protein 1	1434019_at	0.006	0.0019	-1.89
peptidyl prolyl isomerase H	1431506_s_at	0.029	0.0052	-1.55
<b>phosphatidylethanolamine binding protein 1</b>	<b>1415950_a_at</b>	<b>0.003</b>	<b>0.0051</b>	<b>-2.03</b>
phosphatidylethanolamine binding protein 1	1438649_x_at	0.003	0.0031	-1.71
phosphatidylinositol glycan anchor biosynthesis, class Y	1428556_at	0.001	0.0007	1.96
PIH1 domain containing 1	1447750_x_at	0.019	0.0037	-1.21
polyhomeotic-like 2 (Drosophila)	1437239_x_at	0.036	0.0064	1.44
<b>predicted gene, ENSMUSG00000052976</b>	<b>1444178_at</b>	<b>0.006</b>	<b>0.0021</b>	<b>-2.03</b>
<b>predicted gene, ENSMUSG00000076577</b>	<b>1452463_x_at</b>	<b>0.049</b>	<b>0.0088</b>	<b>4.80</b>
prostaglandin E receptor 2 (subtype EP2)	1449310_at	0.029	0.0049	1.45
protein tyrosine phosphatase, non-receptor type 9	1451037_at	0.034	0.0060	1.51
quinoid dihydropteridine reductase	1437993_x_at	0.031	0.0080	1.90
<b>Ras association (RalGDS/AF-6) and pleckstrin homology domains 1</b>	<b>1434303_at</b>	<b>0.019</b>	<b>0.0036</b>	<b>-3.29</b>
<b>RIKEN cDNA 0710008K08 gene</b>	<b>1424311_at</b>	<b>0.001</b>	<b>0.0047</b>	<b>-2.25</b>
RIKEN cDNA 1200009F10 gene	1429065_at	0.006	0.0018	-1.62
RIKEN cDNA 2410012H22 gene	1428515_at	0.029	0.0055	1.29
RIKEN cDNA 2410018C17 gene	1451118_a_at	0.011	0.0040	-1.39
RIKEN cDNA 2810001A02 gene	1436322_a_at	0.039	0.0072	-1.57
RIKEN cDNA 4833420G17 gene	1431318_at	0.050	0.0089	-1.27
RIKEN cDNA 5031439G07 gene	1435745_at	0.039	0.0071	1.35
RIKEN cDNA 6030442H21 gene	1432746_at	0.019	0.0034	-1.32
RIKEN cDNA 8430419L09 gene	1449936_at	0.036	0.0064	-1.46
S100 calcium binding protein A13	1418704_at	0.047	0.0085	1.41
sal-like 1 (Drosophila)	1450489_at	0.040	0.0074	1.35
solute carrier family 9 (sodium/hydrogen exchanger), member 1	1417397_at	0.034	0.0061	1.14

<b>solute carrier organic anion transporter family, member 2a1</b>	<b>1420913_at</b>	<b>0.010</b>	<b>0.0024</b>	<b>2.47</b>
TatD DNase domain containing 1	1458679_a_at	0.040	0.0086	-1.15
t-complex protein 1	1448122_at	0.037	0.0067	-1.32
tetraspanin 9	1428197_at	0.028	0.0046	1.30
transmembrane protein 93	1417912_at	0.036	0.0066	-1.11
TROVE domain family, member 2	1436534_at	0.047	0.0084	1.65
trypsin domain containing 1	1428689_at	0.037	0.0067	-1.42
ubiquitin carboxyl-terminal esterase L5	1419453_at	0.047	0.0084	1.28
UDP glucuronosyltransferase 1 family, polypeptide A2	1426261_s_at	0.029	0.0053	1.17
yippe-like 5 (Drosophila)	1433593_at	0.011	0.0026	-1.70

**Significant differential expression in the One Way ANOVA only**

---	1420310_at	0.040	0.0138	-1.37
---	1455892_x_at	0.036	0.0121	1.81
<b>alpha fetoprotein</b>	<b>1416645_a_at</b>	<b>0.029</b>	<b>0.0188</b>	<b>-7.20</b>
alpha fetoprotein	1416646_at	0.040	0.0192	-1.96
<b>APOBEC1 complementation factor</b>	<b>1432212_at</b>	<b>0.040</b>	<b>0.0135</b>	<b>-2.36</b>
CKLF-like MARVEL transmembrane domain containing 8	1427964_at	0.047	0.0318	1.45
COMM domain containing 2	1433594_at	0.039	0.0155	-1.58
complement component 3	1423954_at	0.036	0.0266	1.19
<b>enoyl Coenzyme A hydratase domain containing 3</b>	<b>1418862_at</b>	<b>0.040</b>	<b>0.0188</b>	<b>2.11</b>
glycerol kinase	1422703_at	0.040	0.0138	-1.45
<b>histocompatibility 2, class II antigen E alpha</b>	<b>1422892_s_at</b>	<b>0.039</b>	<b>0.0325</b>	<b>5.23</b>
immunoglobulin heavy chain 2 (serum IgA)	1421653_a_at	0.030	0.0153	1.92
immunoglobulin heavy chain 2 (serum IgA)	1425763_x_at	0.030	0.0271	1.88
<b>inositol polyphosphate multikinase</b>	<b>1430031_at</b>	<b>0.040</b>	<b>0.0295</b>	<b>-4.62</b>
microcephaly, primary autosomal recessive 1	1429911_at	0.049	0.0129	1.22
myosin, light polypeptide kinase	1425505_at	0.027	0.0138	-1.52
myosin, light polypeptide kinase	1425506_at	0.040	0.0438	-1.44
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	1417102_a_at	0.036	0.0240	-1.33
p53-associated parkin-like cytoplasmic protein	1427620_at	0.040	0.0136	1.23
PDGFA associated protein 1	1434020_at	0.028	0.0156	-1.34
peptidyl prolyl isomerase H	1424136_a_at	0.043	0.0137	-1.53
RAP2B, member of RAS oncogene family	1448885_at	0.034	0.0164	1.62
RIKEN cDNA 1700041C02 gene	1430190_at	0.046	0.0175	1.81
RIKEN cDNA 1700041C02 gene	1456557_at	0.039	0.0145	1.29
schlafen 2	1450165_at	0.040	0.0110	1.29
solute carrier organic anion transporter family, member 2a1	1450032_at	0.037	0.0294	1.81
suppression of tumorigenicity 5	1428372_at	0.049	0.0111	1.62
thymopoietin	1426349_s_at	0.031	0.0119	-1.29
transducin (beta)-like 1 X-linked	1434643_at	0.039	0.0181	-1.75
translin	1448516_at	0.034	0.0180	-1.32

UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4	1423637_at	0.048	0.0620	-1.31
very low density lipoprotein receptor	1438258_at	0.029	0.0114	1.61

**Significant differential expression with Nested ANOVA only**

apoptosis, caspase activation inhibitor	1452996_a_at	0.055	0.0096	-1.19
ATPase, class II, type 9A	1415932_x_at	0.058	0.0099	-1.48
ATPase, class V, type 10A	1452013_at	0.055	0.0094	-1.28
glutamate receptor, ionotropic, N-methyl D- aspartate-associated protein 1 (glutamate binding)	1436297_a_at	0.055	0.0094	1.51
<b>Immunoglobulin heavy chain 6 (heavy chain of IgM)</b>	<b>1427756_x_at</b>	<b>0.051</b>	<b>0.0090</b>	<b>2.04</b>
<b>myelin protein zero-like 2</b>	<b>1416237_at</b>	<b>0.058</b>	<b>0.0099</b>	<b>2.03</b>
regulator of G-protein signaling 3	1425296_a_at	0.055	0.0095	1.36
RIKEN cDNA 1810008I18 gene	1459141_at	0.055	0.0094	-1.76
RIKEN cDNA 4632419I22 gene	1433954_at	0.058	0.0098	1.40
RIKEN cDNA C730049O14 gene	1435084_at	0.055	0.0095	1.58
synaptophysin-like protein	1435081_at	0.058	0.0099	-1.14
trypsin domain containing 1	1441856_x_at	0.058	0.0099	-1.37
v-raf murine sarcoma 3611 viral oncogene homolog	1428607_at	0.055	0.0094	-1.30

Gene Title	Affymetrix Probeset ID	Nested pval Altitude	One way Adj pval	Log <sub>2</sub> Fold Change
<b>Significant differentail expression in both Two way and Nested ANOVA analyses</b>				
5, 10-methenyltetrahydrofolate synthetase	1460257_a_at	0.0048	0.030	0.68
aldehyde dehydrogenase 16 family, member A1	1447372_at	0.0024	0.011	-0.71
annexin A11	1418468_at	0.0050	0.031	1.25
ATPase, class V, type 10A	1447851_x_at	0.0059	0.038	-0.54
baculoviral IAP repeat-containing 4	1437533_at	0.0017	0.005	-0.42
chemokine (C-C motif) ligand 19	1449277_at	0.0068	0.043	0.61
CKLF-like MARVEL transmembrane domain containing 8	1427964_at	0.0001	0.049	0.56
cytidine deaminase	1427357_at	0.0047	0.006	1.58
cytoplasmic FMR1 interacting protein 2	1428347_at	0.0066	0.039	0.50
desmocollin 2	1421156_a_at	0.0060	0.038	0.43
dihydroorotate dehydrogenase	1417581_at	0.0071	0.044	-0.25
dynein light chain Tctex-type 1	1428116_a_at	0.0041	0.022	-1.51
dynein light chain Tctex-type 1	1453473_a_at	0.0045	0.030	-1.65
dystroglycan 1	1426778_at	0.0046	0.030	0.26
EGL nine homolog 3 (C. elegans)	1418649_at	0.0051	0.030	-1.07
eukaryotic translation initiation factor 3, subunit I	1416233_at	0.0074	0.048	-0.11
expressed sequence AI450326	1442124_at	0.0028	0.012	1.25
glycerol kinase	1422703_at	0.0093	0.048	-0.55
immunoglobulin heavy chain 2 (serum IgA)	1429381_x_at	0.0050	0.031	0.53
<b>immunoglobulin joining chain</b>	<b>1424305_at</b>	<b>0.0055</b>	<b>0.035</b>	<b>2.31</b>
<b>immunoglobulin kappa chain, constant region</b>	<b>1427455_x_at</b>	<b>0.0056</b>	<b>0.036</b>	<b>2.50</b>
<b>immunoglobulin kappa chain, constant region</b>	<b>1427660_x_at</b>	<b>0.0072</b>	<b>0.045</b>	<b>2.22</b>
LMBR1 domain containing 1	1425186_at	0.0061	0.030	0.31
membrane-associated ring finger (C3HC4) 7	1440966_at	0.0060	0.038	-0.37
methylthioadenosine phosphorylase	1424425_a_at	0.0046	0.030	0.62
microcephaly, primary autosomal recessive 1	1429911_at	0.0094	0.038	0.32

motile sperm domain containing 3	1460452_at	0.0033	0.011	-0.28
myosin, light polypeptide kinase	1425505_at	0.0096	0.036	-0.51
neuroblastoma ras oncogene	1422687_at	0.0035	0.023	0.78
OCIA domain containing 1	1424952_at	0.0048	0.030	-0.59
PDGFA associated protein 1	1434019_at	0.0013	0.003	-1.02
PDGFA associated protein 1	1434020_at	0.0001	0.039	-0.42
peptidyl prolyl isomerase H	1431506_s_at	0.0040	0.029	-0.64
phosphatidylethanolamine binding protein 1	1415950_a_at	0.0060	0.003	-0.97
phosphatidylethanolamine binding protein 1	1438649_x_at	0.0030	0.005	-0.75
phosphatidylinositol glycan anchor biosynthesis, class Y	1428556_at	0.0009	0.001	1.02
PIH1 domain containing 1	1447750_x_at	0.0040	0.029	-0.31
predicted gene, ENSMUSG00000052976	1444178_at	0.0025	0.011	-0.96
prostaglandin E receptor 2 (subtype EP2)	1449310_at	0.0060	0.038	0.52
quinoid dihydropteridine reductase	1437993_x_at	0.0071	0.031	0.96
regulator of G-protein signaling 3	1425296_a_at	0.0071	0.044	0.55
RIKEN cDNA 0710008K08 gene	1424311_at	0.0064	0.003	-1.13
RIKEN cDNA 1200009F10 gene	1429065_at	0.0049	0.031	-0.64
RIKEN cDNA 1700041C02 gene	1456557_at	0.0001	0.049	0.37
RIKEN cDNA 1810009N02 gene	1429730_at	0.0001	0.043	-0.34
RIKEN cDNA 2410012H22 gene	1428515_at	0.0043	0.025	0.39
RIKEN cDNA 2410018C17 gene	1451118_a_at	0.0049	0.005	-0.47
RIKEN cDNA 6030442H21 gene	1432746_at	0.0050	0.031	-0.36
RIKEN cDNA 8430419L09 gene	1449936_at	0.0084	0.044	-0.58
RIO kinase 1 (yeast)	1449346_s_at	0.0064	0.039	-0.23
S100 calcium binding protein A13	1418704_at	0.0079	0.029	0.55
schlafen 2	1450165_at	0.0086	0.038	0.38
solute carrier family 9 (sodium/hydrogen exchanger), member 1	1417397_at	0.0076	0.049	0.18
solute carrier organic anion transporter family, member 2a1	1420913_at	0.0026	0.011	1.30
speckle-type POZ protein-like	1459560_at	0.0044	0.030	0.29
tetraspanin 9	1428197_at	0.0061	0.039	0.35
translin	1448516_at	0.0070	0.039	-0.35
UDP glucuronosyltransferase 1 family, polypeptide A2	1426261_s_at	0.0053	0.032	0.23
yippee-like 5 (Drosophila)	1433593_at	0.0024	0.011	-0.78

Unknown	1457267_at	0.0044	0.030	-0.32
<b>Significant differential expression with Nested ANOVA only</b>				
ATPase, class II, type 9A	1415932_x_at	0.0096	0.058	-0.57
ATPase, class V, type 10A	1452013_at	0.0089	0.056	-0.37
cDNA sequence BC038822	1436608_at	0.0083	0.055	0.27
CHMP family, member 7	1451300_a_at	0.0093	0.058	0.30
expressed sequence AA536717	1435686_at	0.0086	0.056	-0.42
F-box and leucine-rich repeat protein 11	1438890_at	0.0085	0.056	0.28
hypothetical protein C730026J16	1437650_at	0.0079	0.051	-0.33
glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	1436297_a_at	0.0099	0.061	0.56
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1425324_x_at	0.0087	0.056	1.56
Immunoglobulin heavy chain 6 (heavy chain of IgM)	1425247_a_at	0.0089	0.056	1.18
<b>immunoglobulin heavy chain 6 (heavy chain of IgM)</b>	<b>1427351_s_at</b>	<b>0.0098</b>	<b>0.060</b>	<b>2.25</b>
mitochondrial ribosomal protein L15	1430798_x_at	0.0093	0.058	-1.92
myelin protein zero-like 2	1416237_at	0.0089	0.056	1.06
NIPA-like domain containing 1	1453345_at	0.0090	0.057	1.98
Notch gene homolog 1 (Drosophila)	1418634_at	0.0084	0.056	0.43
N-terminal EF-hand calcium binding protein 1	1437156_at	0.0094	0.058	-0.78
predicted gene, ENSMUSG00000076577	1452463_x_at	0.0086	0.056	2.13
protein phosphatase 1K (PP2C domain containing)	1452973_at	0.0092	0.058	1.03
RIKEN cDNA 1810008I18 gene	1459141_at	0.0080	0.051	-0.85
RIKEN cDNA 5031439G07 gene	1435745_at	0.0089	0.056	0.44
t-complex protein 1	1448122_at	0.0091	0.057	-0.37
transmembrane protein 93	1417912_at	0.0099	0.058	-0.15
tripartite motif protein 24	1427258_at	0.0099	0.061	1.36
trypsin domain containing 1	1441856_x_at	0.0088	0.056	-0.46
v-raf murine sarcoma 3611 viral oncogene homolog	1428607_at	0.0094	0.058	-0.35
<b>Significant differential expression in the One Way ANOVA only</b>				
alpha fetoprotein	1416646_at	0.0161	0.039	-1.00
<b>alpha fetoprotein</b>	<b>1416645_a_at</b>	<b>0.0191</b>	<b>0.031</b>	<b>-2.87</b>

complement component 3	1423954_at	0.0279	0.039	0.26
enoyl Coenzyme A hydratase domain containing 3	1418862_at	0.0180	0.040	1.16
immunoglobulin heavy chain 2 (serum IgA)	1421653_a_at	0.0129	0.030	0.97
immunoglobulin heavy chain 2 (serum IgA)	1425763_x_at	0.0237	0.030	0.93
<b>inositol polyphosphate multikinase</b>	<b>1430031_at</b>	<b>0.0280</b>	<b>0.043</b>	<b>-2.24</b>
myosin, light polypeptide kinase	1425506_at	0.0285	0.038	-0.50
p53-associated parkin-like cytoplasmic protein	1427620_at	0.0114	0.043	0.31
RAP2B, member of RAS oncogene family	1448885_at	0.0213	0.038	0.69
solute carrier family 35, member C1	1452139_at	0.0100	0.043	-0.40
solute carrier organic anion transporter family, member 2a1	1450032_at	0.0313	0.044	0.85
<b>suppressor of cytokine signaling 2</b>	<b>1449109_at</b>	<b>0.0270</b>	<b>0.049</b>	<b>2.14</b>
transducin (beta)-like 1 X-linked	1434643_at	0.0170	0.044	-0.82
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4	1423637_at	0.0524	0.049	-0.39