# Glucose signaling in naked mole-rats during hypoxia

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# Abstract

In hypoxic conditions, glucose metabolism plays an essential role in energy production in most mammals, as many tissues shift toward anaerobic glycolysis to meet cellular energy demands. However, some hypoxia-tolerant species maintain partial aerobic metabolism or utilize alternative metabolic pathways to sustain ATP production. Of particular interest are mechanisms that regulate metabolic rate and energy balance, as hypoxia suppresses aerobic pathways, potentially leading to cell death in hypoxia-intolerant species. Naked mole-rats (NMRs, *Heterocephalus glaber*) are among the most hypoxia-tolerant mammals and significantly suppress their metabolic rate under hypoxic conditions. NMRs also switch from primarily lipid-based metabolism during normoxia to carbohydrate-based metabolism during hypoxia. Despite their exceptional hypoxia tolerance, how NMRs regulate glucose metabolism and energy balance across developmental stages remains unclear. It is also unknown whether adult NMRs retain neotenic traits that contribute to their hypoxia tolerance, suggesting a potential evolutionary adaptation. The goal of my thesis is to examines how different developmental stages of NMRs regulate their metabolic rate and glucose metabolism in normoxia and hypoxia. We hypothesize that NMRs exhibit abnormal glucose metabolism regulation that varies across developmental stages and caste roles within the colony, influencing their ability to conserve energy under hypoxia. We predict that all groups of NMRs will suppress their metabolic rate and downregulate glucose metabolism pathways to conserve energy, with this suppression being more pronounced in younger animals. To address these questions, newborns, juveniles, and adults (subordinates and queens) were exposed to normoxia (21% $O_2$) or acute hypoxia (7%, 5%, or 3% $O_2$) to measure metabolic rate, body temperature, and blood glucose levels. Glucose, insulin, and insulin-like growth factor-1 (IGF-1) were administered to assess glucose handling in NMRs during normoxia and sever acute hypoxia (3% $O_2$). Molecular

analysis focused on normoxia and severe hypoxia (3% $O_2$) to examine blood lactate levels, glycolytic enzyme activity, glycogen metabolism, gluconeogenesis, IGF signaling, and glucose transporter expression.

Our findings reveal several novel insights into NMR glucose metabolism:

1) All age groups reduce metabolic rate and body temperature across all hypoxia levels. Subordinates and juveniles primarily metabolize lipids in normoxia but switch to carbohydrates in hypoxia, whereas queens rely on carbohydrate metabolism under all conditions.

2) Blood glucose increases in hypoxia across all groups but with differing thresholds. Blood glucose rises in moderate hypoxia for queens and pups but only in severe hypoxia for subordinates and juveniles. While glucose tolerance remains similar across groups in normoxia, clearance times following a bolus glucose challenge are 2-3 times longer in juveniles and subordinates than in queens or pups under hypoxia. Administration of insulin or IGF-1 lower blood glucose in subordinates in all conditions, but only IGF-1 impacts blood glucose in hypoxic queens.

3) Blood lactate accumulation and anaerobic pathways show tissue-specific adaptations. Hypoxia elevates blood lactate levels in all groups, reflecting increased anaerobic metabolism. Gene and protein analyses reveal that subordinates and juveniles enhance glucose mobilization for energy, while queens prioritize ATP conservation for prolonged hypoxic survival.

Overall, these findings highlight metabolic adaptations across tissues, developmental stages, and castes in NMRs, enabling their survival in hypoxia. Subordinates and juveniles exhibit metabolic flexibility, supporting their energy-intensive roles in tunneling, foraging, and caretaking. In contrast, queens prioritize ATP conservation to sustain reproduction. These caste- and age-specific strategies optimize survival and efficiency within the colony, reinforcing the exceptional hypoxia tolerance and resilience of NMRs.

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# Contents

## List of abbreviations

| | |
|---|---|
| **ACC1** | Acetyl-COA Carboxylase 1 |
| **Actb** | Beta-actin |
| **AKT** | Protein Kinase B |
| **AMPK** | AMP-activated protein kinase |
| **AMPK-α2** | AMP-activated protein kinase Alpha 2 |
| **ANOVA** | Analysis of Variance |
| **ATP** | Adenosine Triphosphate |
| **ATPase** | Adenosine Triphosphatase |
| **BAT** | Brown Adipose Tissue |
| **CK** | Creatine Kinase |
| **$CO_2$** | Carbon Dioxide |
| **COPD** | Chronic Obstructive Pulmonary Disease |
| **DNA** | Deoxyribonucleic Acid |
| **DTT** | Dithiothreitol |
| **ERK** | Extracellular signal-regulated kinases |
| **F1P** | Fructose-1-Phosphate |
| **FBPase** | Fructose-1,6-bisphosphatase |
| **$FECO_2$** | Fractional Exhaled Carbon Dioxide |
| **$FIO_2$** | Fractional Inhaled Oxygen |
| **GAPDH** | Glyceraldehyde-3-phosphate Dehydrogenase |
| **G6Pase** | Glucose-6-Phosphatase |

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|---|---|
| **G6PD** | Glucose-6-phosphate dehydrogenase |
| **GH** | Growth Hormone |
| **GK** | Glucokinase |
| **GKRP** | Glucokinase Regulatory Protein |
| **GLUT** | Glucose Transporter |
| **GPX** | Glutathione Peroxidase |
| **GTT** | Glucose Tolerance Test |
| **GYS2** | Glycogen Synthase 2 |
| **HIF** | Hypoxia-Inducible Factor |
| **HK** | Hexokinase |
| **IGF-1** | Insulin-like Growth Factor 1 |
| **IGF-1R** | Insulin-like Growth Factor 1 Receptor |
| **IGF-1TT** | IGF-1 tolerance test |
| **IGF-2** | Insulin-like Growth Factor 2 |
| **IGFBPs** | IGF Binding Proteins |
| **ITT** | Insulin Tolerance Test |
| **KHK** | Fructokinase/Ketohexokinase |
| **KO** | Knockout |
| **LDH** | Lactate Dehydrogenase |
| **LDH-B** | Lactate Dehydrogenase B |
| **MCT** | Monocarboxylate Transporter |
| **mTOR** | Mechanistic Target of Rapamycin |
| **NADH** | Nicotinamide Adenine Dinucleotide (Reduced) |

| | |
|---|---|
| **NADPH** | Nicotinamide Adenine Dinucleotide Phosphate |
| **NMR** | Naked Mole-Rat |
| **NO** | Nitric Oxide |
| **$O_2$** | Oxygen |
| **p-AMPK** | Phosphorylated AMP-activated protein kinase |
| **p-GS** | Phosphorylated Glycogen Synthase |
| **PAPP-A** | Pregnancy-Associated Plasma Protein-A |
| **PCR** | Polymerase Chain Reaction |
| **PCK** | Phosphoenolpyruvate Carboxykinase |
| **PDH** | Pyruvate Dehydrogenase |
| **PEPCK** | Phosphoenolpyruvate Carboxykinase |
| **PFK** | Phosphofructokinase |
| **PFK1** | Phosphofructokinase-1 |
| **PGC-1α** | Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
| **PH** | Potential of Hydrogen |
| **PHKG1** | Phosphorylase Kinase Gamma 1 |
| **PHKG2** | Phosphorylase Kinase Gamma 2 |
| **PI3K** | Phosphatidylinositol 3-Kinase |
| **PK** | Pyruvate Kinase |
| **PYGB** | Glycogen Phosphorylase in Brain |
| **PYGL** | Glycogen Phosphorylase in Liver |
| **PYGM** | Glycogen Phosphorylase in Muscle |
| **qPCR** | Quantitative Polymerase Chain Reaction |

| | |
|---|---|
| **RER** | Respiratory Exchange Ratio |
| **RNA** | Ribonucleic Acid |
| **ROS** | Reactive Oxygen Species |
| **s.e.m.** | Standard Error of the Mean |
| **SGLT** | Sodium-Glucose Linked Transporter |
| **SOD** | Superoxide Dismutase |
| **$T_b$** | Body Temperature |
| **TCA** | Tricarboxylic Acid (Cycle) |
| **UCP1** | Uncoupling Protein 1 |
| **$\dot{V}CO_2$** | Rate of Carbon Dioxide Production |
| **$\dot{V}O_2$** | Rate of Oxygen Consumption |

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# 1.Introduction

## 1.1 Hypoxia

Life on earth operates in constant equilibrium with $O_2$ (oxygen). This vital element fuels cellular processes, powering many biological functions. Yet, this delicate balance can be disrupted by a condition known as hypoxia - a state where the tissues of body are deprived of adequate $O_2$ supply (1). There are several ways in which hypoxia can occur. Environmental hypoxia describes situations where the air itself contains less $O_2$. High altitudes, for instance, have lower atmospheric pressure, leading to decreased $O_2$ availability (2). Similarly, confined spaces or bodies of water with limited $O_2$ exchange can also become hypoxic (3). Underground environments can also have variable $O_2$ levels, with tunnels and mines potentially becoming hypoxic (4, 5). In other instances, even in environments with normal $O_2$ levels, tissues can become hypoxic (6, 7). This can happen due to impaired $O_2$ delivery through the bloodstream. This could be caused by conditions like anemia (reduced blood cells), heart failure (impaired blood pumping) (8), or lung diseases that hinder $O_2$ transfer (e.g., COPD) (9).

The effects of hypoxia can be wide-ranging and depend on the severity and duration of deprivation. Initially, the body of the organism often attempts to compensate for this lack of $O_2$. The respiratory system increases breathing rate and depth to take in more $O_2$ (10-12). Heart rate and blood pressure may also increase to deliver oxygenated blood more efficiently. However, prolonged hypoxia disrupts this delicate equilibrium (13). Cellular processes slow down due to lack of $O_2$, leading to an energy crisis. This can manifest as various symptoms, including rapid breathing, shortness of breath, confusion, and impaired judgment. In severe cases, hypoxia can lead to organ failure, coma and even death (14, 15).

The ability to tolerate hypoxia varies greatly among animals (11). While many species rely on constant $O_2$ intake, making them vulnerable to hypoxic environments, others have evolved remarkable adaptations to survive in low-$O_2$ or even anoxic environments (16, 17). While most mammals can tolerate short-term hypoxia by increasing ventilation frequency and recover in normoxic conditions, some animals can thrive in hypoxic environments due to specific adaptations developed during evolution (18). For example, high-altitude mammals like yaks and Tibetan antelopes have increased lung capacity and hemoglobin (the $O_2$-carrying molecule in red blood cells) concentration, allowing them to extract more $O_2$ from the air (19, 20). Many cetacean and pinniped taxa possess higher tolerances for lower levels of $O_2$ in the blood. These taxa are adept at shunting blood flow away from non-essential organs during dives, prioritizing $O_2$ delivery to the brain and heart (21, 22). Perhaps the most fascinating adaptations come from creatures that live in environments with even lower $O_2$ levels, such as naked mole-rats (NMRs) in underground burrows (23). Here, the intensity and duration of hypoxia can vary greatly, from acute to chronic episodes, or even occur intermittently (13, 23).

Metabolic reprogramming is often a crucial component of physiological adaptations to hypoxia, particularly under changing environmental conditions. This reprogramming frequently leads to shifts in fuel substrate preference, which play a key role in optimizing energy utilization. Patterns of fuel utilization in mammals are primarily driven by the availability and characteristics of carbohydrates, lipids, and proteins as energy substrates. These fuels exhibit distinct properties related to storage capacity, energy density, and efficiency in fueling ATP synthesis (24). For example, carbohydrates are favored during short bursts of intense activity due to their rapid contribution to ATP production, although their low storage capacity within tissues limits their availability. Typically, liver and muscle glycogen, the primary carbohydrate stores, contribute to

less than 5% of total energy reserves (24). In contrast, lipids boast high energy content per unit mass, constituting over 80% of energy reserves of the body (24). This abundance in lipid storage makes lipids ideal for sustaining prolonged low intensity activities. However, their slower contribution to ATP synthesis compared to carbohydrates necessitates their use for activities of longer duration. Finally, while proteins can serve as a fuel source, their oxidation is usually minimized to preserve functional proteins and avoid the metabolic costs associated with nitrogen excretion. Consequently, protein contribution to ATP synthesis during exercise is typically less than 5% (25, 26). In mammals facing environmental stressors such as low $O_2$ availability, fuel utilization predominantly shifts toward carbohydrates and lipids, with minimal reliance on protein oxidation. The balance between carbohydrate and lipid oxidation varies depending on the severity and duration of these stressors (27, 28). This model of fuel selection across mammals suggests that the mixture of fuels used is determined by physiological demands of organism and its ability to manage $O_2$ supply. This model has been validated across various species, demonstrating consistent patterns of fuel utilization in response to $O_2$ availability. Even species with varying aerobic capacities show similar fuel mixtures under specific levels of $O_2$ stress, highlighting the broad applicability of this model (27, 29). Despite data stemming from disparate studies on distantly related species, the generalizability of the mammalian model of fuel utilization is striking. However, deviations from this model have been observed in certain scenarios, such as high-fat diets and gender differences, though the underlying mechanisms remain unclear (30-32).

### 1.2 Glucose metabolism in the body

Glucose, with its chemical formula C6H12O6, is the fundamental monosaccharide sugar within the body, serving as the cornerstone of energy provision for all living organisms (33). It

plays a pivotal role as a substrate in both aerobic and anaerobic metabolic pathways. Specifically, glycolysis, the predominant anaerobic metabolic pathway, initiates the breakdown of glucose into pyruvate and ATP, where pyruvate subsequently serves as a primary substrate for aerobic metabolism (i.e., the Krebs cycle) (34).

In biological systems, organisms often acquire glucose in various forms, including isomeric companions such as fructose and galactose, as well as composite structure like disaccharides lactose and sucrose. Polysaccharides such as starch, glycogen, and cellulose further extend the repertoire of glucose derivatives, providing both nutritional sustenance and structural integrity. Animals, adept at navigating this biochemical landscape, dismantle these complex structures into monosaccharides, storing surplus glucose as glycogen in hepatic and muscular reservoirs for utilization during fasting periods (35).

Glucose metabolism is primarily regulated by the peptide hormones insulin and glucagon, which are produced in the pancreas. These two hormones help regulate the transport of glucose in to and out of the cells. Glucose crosses cellular membranes via specific transport proteins, primarily glucose transporters (GLUTs), which facilitate its uptake from the bloodstream into various tissues (33). There are two kinds of glucose transporters: the first are sodium-glucose linked transporters (SGLTs) that are dependent on ATP and sodium; the second are GLUTs that are sodium and ATP-independent. There are 14 different types of GLUTs but only five have been studied to date (36). Cellular glucose uptake is mainly accomplished via GLUT1 and GLUT4. GLUT1 is an insulin-independent protein responsible for basal glucose uptake, while GLUT4 is insulin-dependent. Insulin increases GLUT4 membrane translocation by different signaling mechanisms. The insulin pathway activates the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway, which acts as a metabolic sensor that responds to insulin stimulation and

incorporates GLUT4 into the plasma membrane. GLUT 2 and GLUT5 transport fructose across the cell membrane, and GLUT5 in particular is highly selective (36, 37). Insulin also plays a significant role in adding blood glucose to glycogen in the liver and muscle tissues (33).

The regulation of glucose metabolism involves systemic mechanisms beyond individual cells, orchestration of glucose dynamics extends beyond cellular borders, encompassing systemic processes of glucose homeostasis. Three primary sources sustain circulating glucose levels: intestinal absorption, gluconeogenesis, and glycogenolysis. In the absorptive state following meals, intestinal absorption reigns supreme, ensuring a steady influx of glucose into the bloodstream. The liver, a central protagonist in glucose metabolism, assumes a pivotal role in both glycogenolysis and gluconeogenesis pathways, ensuring the maintenance of euglycemia even during fasting states. Glycogenolysis facilitates the breakdown of glycogen into glucose monomers, a process exclusive to liver and kidney tissues due to their possession of the glucose-6-phosphatase enzyme. Meanwhile, gluconeogenesis, a metabolic pathway generating glucose from lactate and amino acids, sustains glucose levels during periods of nutrient scarcity (fasting). Thus, the saga of glucose unfolds, a testament to its essential role in the complex coordination of biological processes (38).

### 1.3 Metabolic pathways involved in glucose metabolism

Glycolysis is the first cellular pathway that breaks down glucose, and it includes a series of reactions via which glucose is catabolized. Here, energy is obtained from glucose by converting it into two three-carbon molecules called pyruvates (Fig. 1.3). Glycolysis is an anaerobic pathway, and it occurs in both aerobic and anaerobic organisms. The key role of glycolysis is to generate energy and intermediates for other metabolic pathways. It is a key factor in the regulation of

metabolic rate through the maintenance of ATP, lactate, and pyruvate concentrations, which, in turn, affect other pathways for producing additional ATP (39).

Each reaction in glycolysis is catalyzed by its own enzyme. One of the key regulatory enzymes in glycolysis is phosphofructokinase (PFK1), which catalyzes the conversion of fructose 6-phosphate and ATP to fructose-1,6-bisphosphate. PFK1 also regulates the rate of the glycolysis pathway, which is normally tailored to the energy needs of the cell. Overall, glycolysis converts one six-carbon molecule of glucose into three-carbon molecules of pyruvate. The net products of this process are two molecules of ATP (4 ATP produced - 2 ATP used up) and two molecules of NADH (39, 40).

If $O_2$ is present, pyruvate from glycolysis is converted into acetyl CoA in order to enter the citric acid cycle (33). Critically, in the absence of $O_2$ in the environment, glycolysis usually leads to the accumulation of lactate. Furthermore, under these conditions, only anaerobic mechanisms such as glycolysis can produce ATP. While the amount of glucose used in these pathways is equal to that used in aerobic pathways, the amount of ATP produced is significantly lower (~15-fold). For instance, one mole of glucose yields ~30 moles of ATP in the presence of $O_2$, but only 2 moles ATP in the absence of $O_2$ (34). Thus, in most mammals, the switch from aerobic to anaerobic metabolism for a prolonged period can be a major metabolic stressor that may limit survival.

Conversely, in the presence of $O_2$, accumulated lactate will enter into one of the following pathways (41):

- Oxidation to $CO_2$ and $H_2O$ by the tricarboxylic acid cycle
- Conversion to glucose/glycogen by gluconeogenesis
- Used in protein synthesis

Lactate moves between lactate-producing and lactate-consuming sites via monocarboxylate transporters (MCTs) (Fig. 1.1). There are 14 kinds of MCT isoform in mammals, whereas only MCT1-MCT4 mediate proton-coupled transport of lactate. MCT-1 is found in most cells, while MCT-4 is abundant in the cell membranes of glycolytic tissues. Several factors impact the regulation of MCT secretion. For example, in β-cells of rats, overexpression of MCT and LDH lead to stimulation of insulin secretion (42). Also, expression of MCT-1 is increased in tumor cells with low concentrations of glucose (43). Moreover, expression of MCTs can change with diet. For example, Pierre et al. reported that expression of MCTs in brain cells of mice fed a high fat diet is higher than in mice fed a standard diet (44). Studies have also shown that hypoxia can up regulate the expression of MCT-4 in different tissues in rats and zebrafish (45-47).

When lactate is transported to the liver, it is converted to glucose by the gluconeogenesis pathway. Gluconeogenesis is a metabolic pathway which synthesizes glucose from non-carbohydrate carbon substrates. The main parts of these substrates are lactate, pyruvate, propionate glycerol, and all the amino acids except leucine and lysine. Gluconeogenesis mainly occurs in the liver and kidney with lesser contributions from the small intestine (48). Most enzymes involved in gluconeogenesis are similar to those in the glycolysis pathway except for phosphoenolpyruvate (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase) (33).

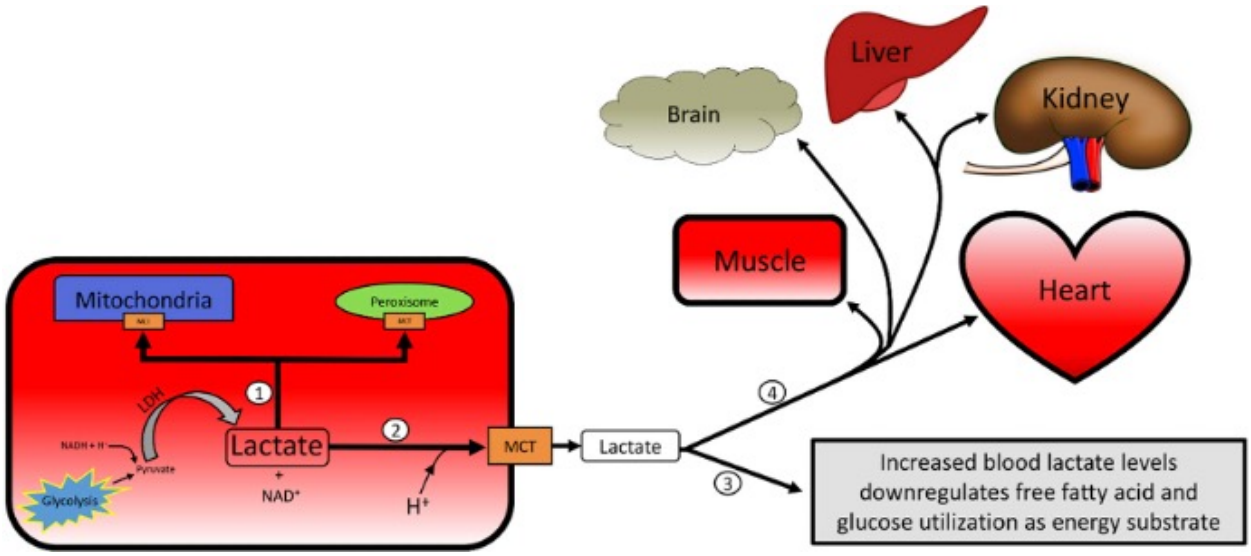


**Figure 1.1 Intracellular and cell-cell lactate shuttle.** 1= intracellular lactate shuttle, where lactate is transported via a monocarboxylate transport protein (MCT) into the mitochondria and oxidized or into peroxisomes for coupled reoxidation of reduced nicotinamide adenine dinucleotide (NADH). 2= Extracellular transport of lactate and one hydrogen ion via MCT. 3= Signaling process by which cellular utilization of free fatty acids and glucose is downregulated. 4= Utilization of lactate as an energy source (Oxidation) by cells in the heart, brain, kidney, liver and other muscles.
(49)

### 1.4 Key hormones regulating [glucose] and the consequences of dysregulated blood glucose

The main function of glucoregulatory hormones is to carefully maintain circulating glucose levels within a limited range. One of the key glucoregulatory hormones in plasma is insulin, which is an anabolic hormone and generated in pancreatic β-cells (34). Insulin, like other hormones, acts by binding to particular receptors, which triggers the sequestration of blood glucose after a meal into skeletal muscle and adipose tissue (see above), where it is stored for future use. At the same time, insulin suppresses endogenous glucose production and glucagon secretion. Simply put, insulin signals the liver to elevate glycogenesis and also stimulates the pancreatic α-cells to suppress glucagon secretion (38, 50).

Glucagon is another key glucoregulatory hormone, and is produced in pancreatic α-cells (34). The main function of glucagon is to elevate blood glucose concentration by increasing the breakdown of glycogen to glucose (glycogenolysis) and stimulating glucose synthesis (gluconeogenesis) (34). Glycogenolysis is the major mechanism that generates glucose during the first 8-12 h of fasting, while gluconeogenesis becomes the principal pathway for glucose production during longer periods of fasting. The key role of glucagon is to facilitate these processes (51).

Disorder in the regulation of blood glucose is the major cause of both diabetes mellitus I and II (52). Specifically, insulin resistance and ultimately, insulin deficiency, are the main reasons for high blood glucose levels observed in diabetes. In type 2 diabetes, insulin is present in the blood, but it is unable to bind to its receptors due to the lower sensitivity of the insulin receptors (52, 53). High blood glucose levels can also lead to osmotic damage to nerves, which leads to peripheral neuropathies and increased inflammation through the reactions that create oxidative stress (54).

### 1.5 Insulin-like growth factor-1 (IGF-1) and carbohydrate metabolism

Insulin-like growth factor-1 (IGF-1) is a 70-amino acid polypeptide hormone, and it is produced primarily by the liver (55). Several studies have demonstrated that IGF-1 plays an important role in the regulation of glucose metabolism. For example, IGF-1 can increase glucose uptake in some peripheral tissues, whereas exogenous injection of IGF-1 decreases blood glucose in both healthy individuals and those with insulin resistance (56, 57). Although the insulin receptor may mediate this response, studies in insulin receptor knockout (KO) mice reported that the hypoglycemic effect of IGF-1 is also mediated by IGF-1R (58).

De Ita et al. demonstrated that there is a reduction in the expression of some genes related to glucose metabolism in mice with partial IGF-1 deficiency (59). Specifically, the expression of phosphoenolpyruvate (PEPCK) and glucose-6-phosphatase (G6Pase), which are two key enzymes in the gluconeogenesis pathway, are reduced in IGF-1 KO mice. PEPCK catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and G6Pase catalyzes the conversion of glucose-6-phosphate to free glucose, which is the last step in gluconeogenesis. These data suggest that activities of IGF-1 are not "insulin-like", instead, it appears that insulin and IGF-1 may have opposed actions. For example, while it is generally accepted that insulin has an essential role in increasing the expression of gluconeogenesis enzymes, this study indicates that disruption of IGF-1 signalling reduces the expression of these enzymes (59, 60). Therefore, these studies support the importance of IGF-1 in glucose metabolism and inform how its deficiency can raise the risk of metabolic diseases.

IGF-1 also has an important role in foetal growth. For example, in mice expressing deletion of IGF-1 and the IGF-1 receptor genes, growth *in utero* is restricted significantly and pups do not survive after birth. Furthermore, these neonates have issues in diaphragmatic muscle development, respiratory failure, and neurologic deficits (61, 62). Notably, the relationship between growth hormone/IGF-1 and insulin is very different in newborn/infant and in children and adults. Growth hormone (GH) is a peptide hormone which is involved in growth, cell reproduction and cell regeneration. Also, GH stimulates IGF-1 generation, which can in turn increase the glucose concentration. Fig. 1.2 shows that, although growth hormone has a key role in IGF-1 concentration, insulin is also involved in this pathway, while in the preterm newborn infant, insulin plays a significant role in IGF-1 generation (63).

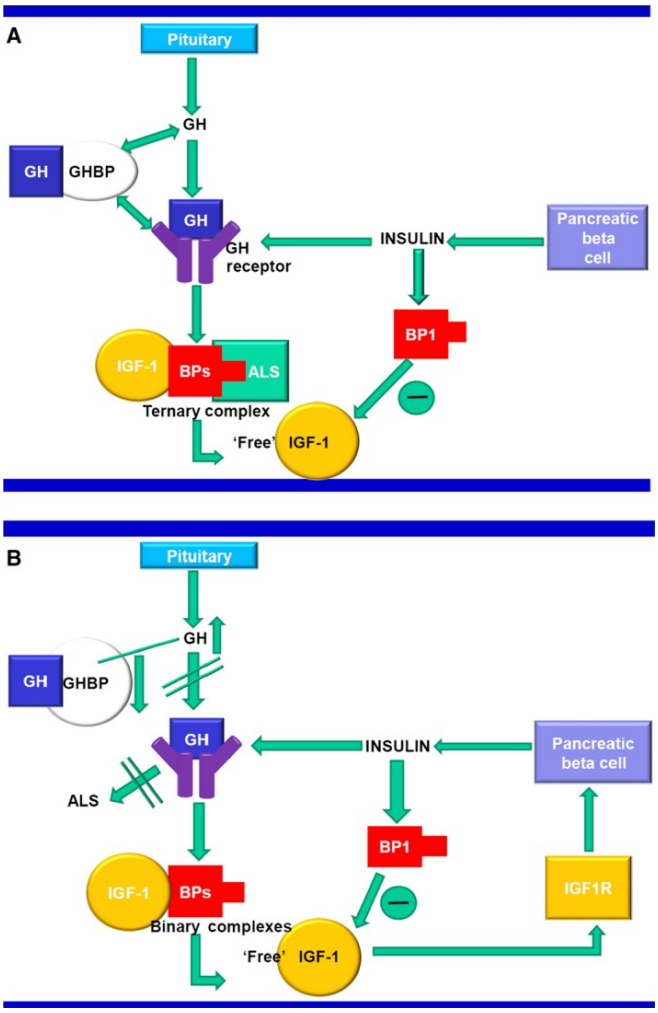


**Figure 1.2 Schematic describing the relationship between insulin and the growth hormone (GH)/IGF-1 axis in (A) childhood and in (B) preterm newborn** (63).

## 1.6 Effects of hypoxia on glucose metabolism in mammals

$O_2$ is essential for the survival of most animals and reduced $O_2$ availability (i.e., hypoxia) is associated with cell death and organ failure (64). With reductions of $O_2$ in the environment, increased reliance on anaerobic pathways (primarily glycolysis) typically results in the accumulation of lactate in cells and tissues. If this lactate accumulation increases, it can lead to severe damage to all cell types and organs due to progressive tissue acidosis (41, 65).

Studies have shown that hypoxia can induce changes in blood glucose concentration and related hormones in mammals. In addition, several studies have illustrated that hypoxia can lead to insulin resistance in some species. For example, insulin resistance is observed in newborn calves after 2 hr hypoxia exposure (66), and also in genetically obese mice during chronic hypoxia (12 weeks) (67). Furthermore, insulin secretion is increased in newborn mice exposed to hypoxia (12% $O_2$) (68), while insulin sensitivity is reduced markedly in healthy men and women following chronic sustained hypoxia at high altitude (69, 70). Conversely, hypoxia can increase insulin sensitivity in some mammals. For example, insulin sensitivity is increased in mice breathing 10% $O_2$ for 4 weeks (71), in dogs in 8% $O_2$ (72), and in rats during anoxia (73, 74).

### 1.7 Fructose metabolism

Fructose differs from glucose in its initial phosphorylation step upon cell entry, leading to distinct downstream metabolic pathways (75). Fructose metabolism bypasses key regulatory steps in glycolysis, promoting enhanced lipogenesis and reducing fatty acid oxidation. Moreover, fructose metabolism generates metabolites with potential implications for metabolic disturbances, such as glycerate and glycerol-3-phosphate, which contribute to triglyceride and phospholipid synthesis (76, 77). Fructose metabolism also leads to rapid reduction in intracellular phosphate and ATP levels, triggering a cascade of events including AMP deaminase activation, uric acid production, mitochondrial oxidative stress, and inhibition of the TCA cycle enzyme aconitase (78-80). This cascade ultimately stimulates de novo lipogenesis, hepatic fat accumulation, and metabolic syndrome. These findings highlight the detrimental effects of excessive fructose consumption on metabolic health, underscoring the importance of understanding fructose metabolism in addressing metabolic diseases.

Fructose metabolism is particularly crucial during hypoxia for maintaining active glycolysis. The regulatory enzyme PFK1 plays a crucial role in glycolysis, being allosterically inhibited by ATP, citrate and lactate (81, 82). During hypoxia, lactate accumulation inhibits PFK1, slowing down glycolytic flux and limiting ATP and glycolytic intermediates. Fructose metabolism, however, circumvents this inhibition by utilizing the enzyme fructokinase (KHK) to phosphorylate fructose to fructose-1-phosphate (F1P), which enters glycolysis downstream of PFK1. This unchecked fructose metabolism ensures continuous ATP and glycolytic intermediates supply, benefiting cells under hypoxic conditions (Fig. 1.3) (83). Also, the utilization of fructose in tumor cells indicate that the rapid kinetics of the KHK enzyme and its lack of regulation lead to a significant depletion of ATP during the initial phosphorylation of fructose upon cell entry (84). This depletion briefly relieves the inhibition of PFK1, enhancing the flow of glycolysis from glucose to generate ample ATP and glycolytic intermediates for cellular growth and maintenance (77, 85, 86). Additionally, in hepatocytes, F1P alleviates the suppression of glucokinase (GK) by Glucokinase regulatory protein (GKRP), thereby activating GK and facilitating the phosphorylation of glucose for entry into glycolysis or gluconeogenesis (87, 88).

Excessive consumption of fructose is associated with metabolic syndrome in both humans and animals (89). Fructose-rich diets are consumed by various species, including migrating birds (90), bears (91, 92), and mouse lemurs (93, 94), as a means to store energy for survival during periods of food scarcity. Fructose consumption promotes fat and glycogen accumulation, potentially aiding in hydration due to the water content of these molecules (86). Animals facing environmental stresses like heat or dehydration activate fructose production via the polyol pathway, contributing to fat accumulation (95). Fructose-induced insulin resistance may serve as

an adaptive defense mechanism against food shortages, allowing for prioritization of glucose for essential organs like the brain (96-98).

### 1.8 Hypoxia in hypoxia-tolerant animals

In most mammals, when cells are deprived of $O_2$ due to environmental or systemic hypoxia, aerobic metabolism slows or stops, and they must rely upon anaerobic metabolism to balance the gap between energy demand and energy supply (12, 17, 99). This approach is usually effective for short-term or mild hypoxia, but is problematic in prolonged or severe hypoxia due to the deleterious accumulation of lactate derived from the glycolysis pathway, the metabolization of which requires $O_2$ (48). Although such a hypoxic environment is energetically challenging for animals, some species nevertheless inhabit and thrive in these niches. Indeed, hypoxia is a common challenge for animals that live in underground burrows or caves (e.g., African mole-rats, bats) or animals that inhabit high altitude niches that experience hypobaric hypoxia (e.g., *Peromyscus maniculatus* – high altitude deer-mice, *Eospalax fontanierii baileyi* – plateau zokors, among others) (19, 100-103). It is believed that living long-term in a hypoxic environment drives the evolution of cellular and physiological traits in these animals that enhance tolerance to low $O_2$ stress (18). There are some common strategies that many hypoxia-tolerant mammals employ to tolerate a hypoxic environment. The most common response to low $O_2$ availability in hypoxia-tolerant species is a robust decrease in metabolic rate (17, 104), which reduces overall energy demand. The key strategies that hypoxia-tolerant animals employ to suppress their metabolic rate include a coordinated downregulation of energy metabolism (TCA cycle, glycolysis, and β-oxidation (105-107)), and also of cellular consumers of ATP such as ion pumps (108).

These downregulation in energy metabolism can lead to elevation in blood glucose concentrations in some species, such as the frog, *Rana catesbeiana* (109), Andean mice (110), and some species of freshwater turtles (111). The expression of some genes associated with energy metabolism and blood glucose concentrations are altered by hypoxia in hypoxia-tolerant animals (100, 112). Also, genetic analysis of PFK (a key enzyme in the glycolysis pathway) indicates that there are sequencing differences between hypoxia-tolerant and hypoxia-intolerant animals (100). Importantly, many of the most anoxia-tolerant species have also developed unique lactate management strategies that help to mitigate the deleterious impact of anaerobic metabolism. For example, carp, goldfish, and western painted turtles are among the most anoxia-tolerant vertebrates and are able to maintain their tissue ATP at a constant level during anoxia and avoid metabolic acidosis (113, 114). Carp and goldfish have evolved a mechanism to convert lactate into ethanol, which they are able to excrete across their gills (115), whereas painted turtles sequester protons in their bones and shells to mitigate metabolic acidosis in prolonged anoxia (113). Indeed, although these animals rely entirely on anaerobic pathways during anoxic conditions, an $O_2$ debt is not observed, and lactate oxidation does not occur, while glycogen synthesis increases significantly to support anaerobic pathways (113, 115-117).

### 1.9 Ontogeny of carbohydrate metabolism in NMRs in normoxia and hypoxia

NMRs are one of the most hypoxia-tolerant mammals. They live in crowded burrows and are the longest living rodent known (118). They are not only resistant to cancer and certain damaging effects associated with aging (119), but they can also tolerate harsh environments such as hypoxia that most other species cannot. Under laboratory conditions, they are able to tolerate <

3% $O_2$ for several h (120), and 8-10% $O_2$ for days to weeks (121), and they can withstand anoxia for up to 18 min (122).

Several studies have demonstrated that NMRs are able to reduce their metabolic rate during hypoxia to tolerate hypoxic conditions. For example, they decrease their metabolic rate by up 85% in 3% $O_2$ (120, 123). It is not exactly clear how they suppress their metabolic rate, but NMRs reduce their activity and thermogenesis (124, 125) to save energy, among other potential mechanisms. Metabolic rate suppression in NMRs occurs after just a few min of exposure to hypoxia. Indeed, they are able to suppress their metabolic rate by 75% within 90 seconds of anoxia (125). Importantly, NMRs respond quickly to environmental changes: when they enter into normoxia following anoxia, their physical activity resumes to the pre-anoxia levels within 5 min (125). Investigation of metabolic rate in different hypoxic environments with varying levels of $O_2$ exposure may help us understand whether there is any relationship between the level of metabolic rate suppression and type of substrate metabolized.

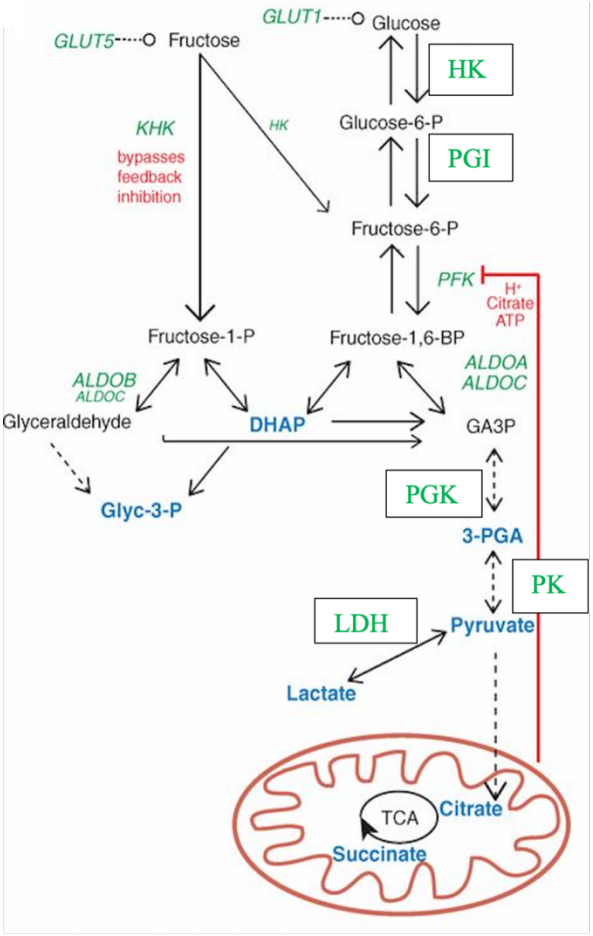


**Figure 1.3 Glycolysis pathway.** Glucose enters the brain via GLUT 1 and is converted via phosphofructokinase (PFK). Fructose enters cells via GLUT5 and is phosphorylated by ketohexokinase (KHK) to fructose-1-phosphate (F1P) at a much higher efficiency than by hexokinase (HK) (122)

Changes of blood glucose concentration in NMRs during hypoxia may be an important factor for hypoxia adaptation in this species. NMRs are able to increase their blood glucose concentration during hypoxia and anoxia (123). To investigate the fuel switching following hypoxia, Pamenter et al. found that when blood glucose concentration increases (roughly doubled), liver glucose levels decrease significantly at the same time. These data suggested that mobilization of liver glucose in hypoxia support blood glucose in hypoxia (Fig. 1.4). It is not clear how and why NMRs use this mechanism during hypoxia. Therefore, measuring blood glucose of NMRs in

various $O_2$ levels and at a different time during hypoxia can help us to understand the impacts of blood glucose changes on their hypoxia adaptation.

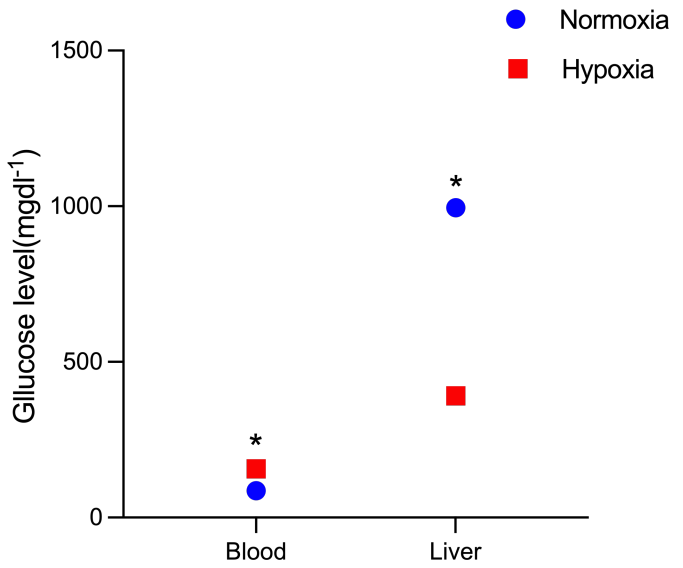


**Figure 1.4 Glucose concentration in NMR during hypoxia.** Mobilization of liver glucose in hypoxia support blood glucose. Data are presented as mean ± SEM. (*) indicates s significant difference ($P<0.05$) between normoxia and hypoxia groups. (123)

The response of NMRs to insulin is also different than that of mice. Kramer et al. injected glucose and insulin into NMRs and mice to investigate how they regulate blood glucose levels after injection. Pre-injection blood glucose concentrations for both saline and glucose injected animals were similar. Following saline injection, no significant change in blood glucose level was evident. Interestingly, after glucose injection, they observed that the blood glucose level of NMRs started to increase after 15 min and reached a peak value (exceed 200 mg/dl) 45 ± 12.2 min post-injection and returned to the baseline values 156 ± 31 min later. The blood glucose concentration changes in mice were different, reaching a greater peak value (350 mg/dl) after 15 min and returning to the baseline level after 1 hr. These results suggest that NMRs are insulin resistant relative to mice (Fig. 1.5). In another experiment, when insulin was injected into NMRs and mice,

the blood glucose level of NMRs dropped markedly and after around 1 hour decreased below 30 mg/dl, whereas changes were slower in mice and their blood glucose level returned to baseline values after 1 hr. Confusingly, these rapid changes in blood glucose concentration of NMRs suggest that they are sensitive to insulin (Fig. 1.5). Therefore, it is not clear whether NMRs are sensitive or resistant to insulin (126).

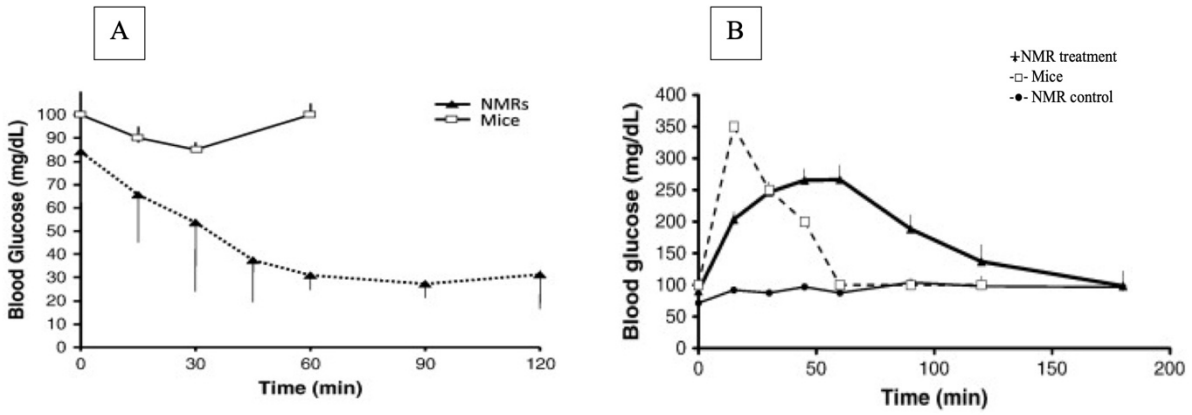


**Figure 1.5 Blood glucose in NMRs and mice following intraperitoneal injection with insulin and glucose.** NMR blood glucose drops rapidly following insulin injection and remains low for a prolonged time compared to mice (A) Blood glucose in NMRs and mice following glucose injection (B), blood glucose remains elevated for longer periods in NMRs compared to mice. NMRs injected with saline were used as control (B) (n = 10 for each group). Data represent means ± standard deviation (126).

NMRs exhibit a unique metabolic strategy for surviving extremely low $O_2$ conditions, including anoxia, which is the complete absence of $O_2$. Studies using advanced techniques revealed significant increases in fructose and sucrose levels in NMRs compared to mice under anoxic conditions (122). This suggests that unlike mice, NMRs accumulate fructose to potentially fuel vital organs like the brain and heart in anoxia. By contrast, studies on the hypoxic brain (7% $O_2$ for 4 h) showed that the expression of GLUT5 and ketohexokinase (KHK) is downregulated, while lactate dehydrogenase (LDH) is upregulated, indicating a shift toward glucose metabolism (127). Fructose metabolism offers several advantages to NMRs during anoxia. Firstly, it bypasses the key

regulatory checkpoint of glycolysis, phosphofructokinase-1, which gets suppressed by factors like cellular acidification, a common consequence of anaerobic metabolism (128). This allows NMRs to continue using glycolysis for ATP production even when cellular activity is high. Secondly, gene expression analysis indicates a predisposition for fructose metabolism in NMRs, possibly due to higher levels of fructose-metabolizing genes (122). This suggests that NMRs might even synthesize fructose internally under hypoxic conditions. Brain slices from NMRs displayed maintained synaptic activity when fueled by fructose, although not as effectively as with glucose (122). In anoxic environment, stable isotope tracing confirmed efficient fructose utilization in NMRs brain tissue, highlighting its metabolic adaptation to $O_2$ deprivation. Interestingly, the NMR heart also showed resilience when fueled by fructose, unlike the mouse heart (122).

The preference of NMRs for fructose as a substrate in vital tissues during anoxia parallels other biological systems that face extreme environmental pressures as a survival strategy. For example, cancer cells exhibit metabolic plasticity, often relying on fructose metabolism to meet their high energy demands for growth in $O_2$-deprived environments (129). In various cancer types, the expression of GLUT5 and aldolase B is elevated, enhancing fructose metabolism, which in turn promotes tumor growth and the spread of cancer cells (130-133). Similarly, in cardiac ischemia models, fructose metabolism is activated as a last-ditch attempt to survive, driving anabolic growth of the myocardium to meet energy and biosynthetic demands under hypoxia (134). Fructose metabolism is also observed in neonatal hearts during the proliferative phase, suggesting its importance in promoting cardiomyocyte proliferation and regeneration (135, 136). Additionally, fructose plays a vital role in fetal growth and development in mammals like whales and pigs, activating cell signaling pathways critical for placental and fetal development (137, 138). The ability of NMRs to utilize fructose may reflect its retention of neonatal traits into adulthood,

providing phenotypic flexibility during evolution (139-141). These biological systems share common features of low $O_2$ environments and cellular proliferation demands. Enhanced fructose metabolism under these conditions supports ATP and biosynthetic needs when oxidative metabolism is limited (122).

Colonies of NMRs typically include one breeding queen, multiple breeding males, and many pre-pubertal subordinates (142). There is a social hierarchy within the colony, which is associated with body weight (143). It has been suggested that adult NMRs may sustain a neonatal phenotype into adulthood, which may contribute to their hypoxia-tolerance (139, 144). Indeed, many features observed in NMRs, such as metabolic rate suppression during hypoxia, are consistent with traits retained from their neonatal period (145). Furthermore, although NMRs are poor thermoregulators, they do have brown adipose tissue (BAT), which is a key player in body temperature regulation in homeothermic mammals (146). Oiwa et al. illustrated that NMRs express BAT with normal thermogenic activity. They observed that NMRs-BAT thermogenesis slightly delays the decrease in the body temperature at temperature 20 $^{O}$C (a cold temperature for NMRs), but it cannot avoid decreasing body temperature overall, likely due to the poor insulator characteristics of naked NMR skin. Interestingly, these authors also found that at temperature 30$^{O}$C, NMRs are able to maintain their body temperature through social/group heat-sharing behaviour in the colony, but in isolation, only queen NMRs are able to maintain their body temperature (147). Additionally, our previous research demonstrated that during hypoxia, non-shivering thermogenesis in NMRs is actively suppressed, allowing them to lower their body temperature and conserve energy. This suppression is directly linked to a sharp decrease in uncoupling protein 1 (UCP1) activity in BAT, which, under normoxic conditions, plays a crucial role in facilitating heat production. In normoxic environments, NMRs depend on UCP1-mediated

thermogenesis to maintain body temperature. However, when exposed to hypoxia, UCP1 levels drop significantly, leading to diminished mitochondrial activity in BAT and a subsequent reduction in heat production. This adaptive response allows NMRs to significantly reduce both metabolic rate and body temperature, enhancing their survival under low $O_2$ conditions (124).

Additionally, glucose is the primary fuel substrate for BAT, and these data show that NMR queens are better able to maintain body temperature in challenging environments, suggesting variations in glucose metabolism and BAT function between different NMRs based on development, sex, or social status.

### 1.10 Study hypothesis

The main hypothesis of this study is that NMRs have abnormal glucose metabolism regulation that differs across development and by caste within the colony. As described above, our knowledge of glucose metabolism in NMRs is very limited, and nothing is known about the effects of hypoxia on this system. In this thesis, I aimed to explore different aspects of glucose metabolism in NMRs throughout their development to better understand this aspect of their tolerance to environmental hypoxia. NMRs, including adult subordinates (1-12 years old), queens (3-12 years old), juveniles (two months old), and pups (2-10 days old), were used in these experiments. Both sexes of animals were used equally in this study. Animals were group-housed on a 12 h: 12 h light: dark cycle in their native habitat (30°C; 70% relative humidity). My project is divided into three chapters, as follows:

Chapter 2 – Comparing the impact of acute hypoxia on metabolic rate and blood glucose levels throughout developmental stages of NMRs. The first data chapter of my proposal evaluated the physiological responses of NMRs to hypoxia and glucose regulation. Experiments included

measuring metabolic rate and blood glucose levels from NMRs at each developmental stage in both normoxia and hypoxia. I also injected glucose, insulin, or IGF-1 into NMRs to observe how they regulated their blood glucose and to assess the role of these hormones in mediating changes in blood glucose under normoxia and hypoxia. The purpose of this chapter was to determine whether there were any differences in metabolic rate and blood glucose levels in NMRs across different $O_2$ levels and developmental stages, and how these changes were regulated.

Hypothesis: Acute hypoxia would differentially impact metabolic rate and blood glucose levels in NMRs at various developmental stages, with younger NMRs showing greater adaptability. Glucose, insulin, and IGF-1 would modulate these responses, varying with developmental stage.

Prediction: Our predictions included: 1) younger NMRs would reduce metabolic rate and maintain blood glucose levels more effectively under hypoxia compared to older NMRs, 2) glucose administration would raise blood glucose levels, especially in younger NMRs, 3) insulin would lower blood glucose more effectively in younger NMRs, particularly in normoxia, and 4) IGF-1 would enhance glucose regulation in hypoxia, particularly in younger NMRs.

Chapter 3– Evaluating blood lactate levels and anaerobic pathways across developmental stages of NMRs in normoxia and acute sever hypoxia. In the second data chapter of my thesis, I will assess blood lactate levels, as well as the activity and expression of key enzymes and proteins involved in these pathways, in both normoxia and hypoxia (3% $O_2$ for 1 h) in juvenile, subordinate, and queen NMRs.

Hypothesis: We hypothesized that the significant metabolic rate suppression observed in hypoxic NMRs would involve a shift away from $O_2$-dependent pathways, resulting in increased reliance on anaerobic metabolism.

Prediction: We predicted that 1) glycolytic enzyme activity would either remain unchanged or decrease in peripheral tissues, 2) enzymes involved in glycogen metabolism and gluconeogenesis, which depend on $O_2$, would be downregulated to conserve energy, and 3) lactate transport proteins would be downregulated, potentially resulting in lactate accumulation in the blood despite minimal activation of glycolytic pathways. Additionally, we predicted that younger animals would show a greater reliance on anaerobic pathways due to developmental stage.

Chapter 4 – Investigating the role of IGF signaling pathways in glucose regulation during severe hypoxia across key tissues and developmental stages in NMRs. In the second data chapter of my thesis, I measured the expression of IGF-1, IGF-2, their receptors, the insulin receptor, and glucose/fructose transporters in animals exposed to either normoxia or severe hypoxia (3% $O_2$ for 1 hr).

Hypothesis: We hypothesized that severe acute hypoxia reduces IGF expression to limit glucose uptake in most tissues, aiding in metabolic rate suppression.

Prediction: Our predictions included: 1) a decrease in IGF-1 and IGF-2 expression in the liver, muscle and kidney, and 2) a downregulation of insulin and IGF receptors, along with glucose/fructose transporters, in the same tissues to conserve energy. Additionally, we predicted that this downregulation would be more pronounced in younger animals due to their greater developmental energy demands.

### 1.11 Significance

Exploring glucose metabolism in NMRs offers a number of advantages. First, understanding how NMRs regulate their energy metabolism during hypoxia will help us to grasp the complex processes of evolution in hypoxia-tolerant animals driven by life in low $O_2$

environments. Secondly, investigation of glucoregulatory pathways in NMRs may play an important role in the development of novel approaches to treat pathologies related to hypoxia, such as chronic pulmonary and cardiac disorders, anemia, diabetes, and stroke. More importantly, the investigation of glucose metabolism in different developmental stages of NMRs can clarify the neotenic theory of hypoxia-tolerance. Additionally, this study may provide insight into how social structure and reproductive roles influence metabolic adaptations during hypoxia in NMRs.

# Chapter 2: Hypoxia impairs blood glucose homeostasis in naked mole-rat subordinate adults but not queens

This chapter includes material from the following article:

Ojaghi, M., & Pamenter, M.E. (2024). Hypoxia impairs blood glucose homeostasis in naked mole-rat subordinate adults but not queens. The Journal of Experimental Biology, 15;227(10). https://doi.org/ 10.1242/jeb.247537

## 2.1 Introduction

NMRs often experience fluctuating $O_2$ levels in their densely populated underground burrows, which necessitates physiological adaptations that enable survival in hypoxic burrows (13, 23). As discussed in chapter 1, subordinate NMRs, which make up the non-reproductive majority of the colony, can drastically reduce their metabolic rate and $T_b$, and switch from lipid-based metabolism under normoxic conditions to carbohydrate-based metabolism during hypoxia (121, 123, 124, 148, 149). This metabolic flexibility is believed to be a key factor in their hypoxia tolerance. However, while the metabolic adaptations of subordinate NMRs have been investigated in various studies, there is limited understanding of how metabolic rate and blood glucose regulation, both critical aspects of energy metabolism, vary across developmental stages and between different social castes within the colony, particularly in breeding animals like queens.

Glucose homeostasis in adult mammals is primarily mediated by insulin and IGF-1, which help maintain stable blood glucose levels by promoting glucose uptake and storage in tissues like muscle and fat (50). Insulin and IGF-1 act through their respective receptors to mediate these effects, but some studies suggest that NMRs may exhibit unique responses to these hormones (126, 150). For example, unlike mice, NMRs have been shown to display insulin resistance following glucose injection, while they exhibit rapid drops in blood glucose following insulin injection. These conflicting responses make it difficult to clearly characterize the insulin response of NMRs (126). This paradox highlights the need for further investigation into the regulation of glucose metabolism in this species, particularly in the context of hypoxia, where energy demand, and glucose utilization are likely to shift dramatically.

To address these gaps, this chapter explored the impact of hypoxia on blood glucose regulation and the roles of insulin and IGF-1 signaling in this regulation throughout development.

We hypothesized that NMRs rapidly mobilize glucose to help maintain anaerobic carbohydrate metabolism in hypoxia. We predicted that this response is similar across developmental stages, indicating retention of neotenic traits into adulthood (151) and that IGF-1 plays a more important role in blood glucose regulation than insulin. To test our hypothesis, we exposed NMR pups (~ 2-10 days old), juveniles (~ 8 weeks old), and adult subordinates (~ 1-13 years old), and queens (~ 3-12 years old) to 1 hr of normoxia (21% $O_2$), followed by 1 hr of hypoxia (7%, 5% or 3% $O_2$), while indirectly measuring metabolic rate via respirometry. Blood glucose and $T_b$ were also measured. In other experiments, we challenged NMRs with bolus injections of glucose, insulin, or IGF-1 in normoxia or hypoxia to evaluate the underlying mechanisms regulating blood glucose homeostasis and how these are impacted by environmental $O_2$ availability in a hypoxia-tolerant mammal.

## 2.2 Materials and Methodology

### *2.2.1 Animals and ethics*

All experiments were approved by the University of Ottawa Animal Care Committee and conducted in accordance with the Animals for Research Act and by the Canadian Council on Animal Care. NMRs were bred in the animal care facility at the University of Ottawa and housed in groups in multi-cage systems maintained at 21% $O_2$, 30°C, 70% humidity and a 12 h:12 h dim light:dark cycle. To mitigate potential effects of circadian variation, all experimental procedures were conducted at the same time of day. Previous studies showed that NMRs retain a functional but weak circadian system. While they live in constant darkness and have poor vision, they still show daily rhythms in metabolism, body temperature, and activity. Their core clock genes are intact but show unusual expression patterns (152). Animals were fed fresh tubers, vegetables, fruits, and Pronutro cereal supplement *ad libitum*. Animals were not fasted before experimental trials. 229 NMRs were used in this study, including 55 adult subordinates (1-13 years old; 55 ± 10 g), 8 queens that had previously had successful litters but that were not pregnant or lactating (3-12 years old; 65 ± 8 g), 7 juveniles (~2 months old; 13 ± 2 g), and 159 pups (2-10 days old; 2.5 ± 0.5 g). To avoid inbreeding effects and reduce the risk of pseudoreplication, animals were selected from multiple independent colonies. We maintained an equal representation of both sexes for the subordinate animals, but we were not able to determine sexes of pups and juveniles. For the queen and juvenile groups, the same animals were used in all experiments due to a limited number of available animals at these developmental stages. Animals were allowed a minimum of a two-week interval between repeated measurements to allow for adequate recovery. For subordinates, different animals were used for each experiment.

### *2.2.2 Whole body respirometry*

A 450 ml Plexiglass chamber was set inside a larger, thermally controlled chamber, which was in turn held at ~ 28°C. Animals were placed individually inside of the smaller chamber and their $T_b$ was recorded every 10 min using previously implanted RFID microchips (840 Lifechip 134 KHZ BT Domestic Equine, Destron Fearing, Langeskov, Denmark) and an RFID microchip reader (Allflex Inc., Dallas, TX). To reach the desired $O_2$ level, the animal chamber was sealed and ventilated with gas mixtures set by calibrated rotameters (Krohne, Duisburg, Germany). This experimental paradigm is based on extensive pilot studies with longer exposures in hypoxia (123, 125). The flow rate ($FR_I$) of gas into the animal chamber was set at 100 ml/min using a calibrated mass flow meter (Q-G265, Qubit Systems Inc, Kingston, ON, Canada). Pups were too small to effectively measure metabolic rate in this system, or to implant RFID microchips, and so were not included in whole body respirometry experiments.

Analyzers were calibrated before each experiment using premixed gases containing 20.95% $O_2$ and 1.5% $CO_2$, or 100% $N_2$. Respirometry data were recorded by a Q-S102 $O_2$ analyzer and a Q-S153 $CO_2$ analyzer (Qubit Systems Inc, Kingston, ON, CAN). To record the rates of $O_2$ consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$), the outflow and the inflow gas concentrations were compared. To calculate $\dot{V}_{O_2}$ (ml $min^{-1}$ $kg^{-1}$), equation 10.6 in Lighton (2008) was used (153):

$$\dot{V}_{O_2} = FR_I\,[(FI_{O_2} - FE_{O_2}) - FE_{O_2}\,(FE_{CO_2} - FI_{CO_2})]/(1 - FE_{O_2}).$$

$\dot{V}_{CO_2}$ was calculated using equation 10.7:

$$\dot{V}_{CO_2} = FR_I\,[(FE_{CO_2} - FI_{CO_2}) - FE_{O_2}\,(FI_{O_2} - FE_{O_2})]/(1 - FE_{CO_2}).$$

Where $FR_I$ is the incurrent flow rate (ml/min), $FI_{O_2}$, and $FI_{CO_2}$ are the fractional concentrations of incurrent $O_2$ and $CO_2$ of dry gas, and $FE_{O_2}$ and $FE_{CO_2}$ are the fractional concentrations of excurrent

$O_2$ and $CO_2$ from the experimental chamber, respectively(153). Respirometry Exchange Ratios (RERs) were also calculated by dividing $\dot{V}_{CO_2}$ by $\dot{V}_{O_2}$.

### *2.2.3 Blood sampling and glucose measurements*

Animals were not anesthetized before blood collection. Blood (30 µl) was collected from juvenile or adult NMRs by pricking pedal veins with a 25-gauge hypodermic needle. Blood (30-50 µl) was collected from pups by decapitation using very sharp scissors. The blood glucose concentration was then measured using a blood glucose meter (Contour next one, ASCENSIA, Mississauga, ON, Canada), which has been previously used to assay blood glucose in this species (126).

### *2.2.4 Glucose, insulin, and IGF-1 tests*

Glucose tolerance tests were performed to determine the rate at which animals mobilize systemic responses to a glucose-load injection. We employed an insulin syringe for all injections throughout this study. Briefly, the resting blood [glucose] was measured as described above and then 2 g of glucose (dissolved in distilled water) per kg body weight was injected intraperitoneally (IP). For adults, the concentration of the glucose solution was 0.4 grams per ml, with the injection volume ranging between 0.01 and 0.05 ml, depending on the body weight. In contrast, for pups, the glucose solution concentration was set to 0.2 grams per ml, with the injection volume varying between 0.0015 and 0.003 ml (Volume (ml) = NMR weight (kg) X Dosage(g/kg) / Concentration (g/ml)). Next, for subordinates, queens and juveniles, the blood glucose concentration was measured at 15, 30, 45, 60, 90, 120, 150 and 180 min after glucose injection. Pups were too small to permit repeated blood draws and so individual pups were used to measure blood glucose at each time point. Importantly, we used different pups for blood glucose measurements at each time point; therefore,

the values for total glucose presented for the pup group is an estimation for this developmental stage because it was not possible to conduct repeated sampling in the same individuals. For glucose tolerance tests in hypoxia, animals were first exposed to a hypoxic environment for 1 hr, with baseline blood glucose measurements taken before and after hypoxia. Animals then received a glucose injection and were returned to the hypoxic chamber rapidly after each blood sample. Next, blood glucose levels were measured at 30, 60, 90, 120, 150 and 180 min after injection in hypoxia.

The same procedure was used for both the insulin and IGF-1 tolerance tests. Insulin was diluted 1:1000 in 0.9% NaCl and then was injected IP (0.75 U insulin/kg body weight; human insulin, Gibco, India, 4 mg/ml, Stock: 100 U/mL insulin; working concentration 0.1 U/mL for adults and 0.05 U/mL for pups). Similarly, human IGF-I (Shenandoah Biotechnology, USA; 1 mg/kg body weight; working concentration 0.3 mg/mL) was injected IP to queens and subordinates. After injections or glucose measurements, animals were promptly returned to the chamber, typically within 3 min and the chamber was resealed. We maintained a high flow rate in the chamber during tolerance tests compared to metabolic experiments to ensure a quick return to hypoxia and the chamber $O_2$ level was re-established within 5 min of returning the animal (data not shown).

### *2.2.5 Statistical analysis*

Logger Pro 3 software (Vernier Software & Technology, Beaverton, Oregon, USA) was used to record the incurrent and the excurrent $O_2$, $CO_2$ and $FR_I$ levels. We calculated the average of $T_b$, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$, using the last 10-15 min of each $O_2$ exposure (21%, 7%, 5%, 3% $O_2$). Statistical analysis was performed using GraphPad Prism 9 (GraphPad Prism, La Jolla, CA, USA). All data followed a normal distribution with equal variance ($p > 0.05$). Significant differences ($p < 0.05$)

were determined with one-way ANOVAs and mixed-effects model analysis (REML) to examine intraspecies differences for each independent variable: 1) normoxia, and 2) $O_2$ level (7%, 5% or 3% $O_2$), or 3) glucose, insulin, or IGF-1 injection. When a significance difference was determined, Tukey's or Sidak's multiple comparisons tests were used to identify significantly different normoxia within individual treatment groups. Data are presented as mean ± SEM where $p < 0.05$ was the threshold for significance.

## 2.3 Results

***The hypoxic metabolic response is similar across developmental stages.*** We first evaluated metabolic responses to three different levels of acute hypoxia in juvenile, subordinate, and queen NMRs (Fig. 2.1; note that the small size and sensitivity to isolation of pups prevented us from measuring metabolic responses in this developmental stage). In normoxia, subordinates had the highest metabolic rate (as measured indirectly using respirometry; Fig. 2.1A), followed by queens. The normoxic metabolic rate of juvenile NMRs was ~ 40-50% lower than that of the older animals ($F_{2,58} = 11.7$, $p < 0.0001$ for $\dot{V}_{O2}$, $F_{2,58} = 6.1$, $p < 0.0001$ for $\dot{V}_{CO2}$). With the onset of hypoxia, metabolic rate decreased in all animals and age groups, by ~75-80% in subordinates, ~70-75% in queens, and ~40-60% in juveniles (Fig. 2.1A-D $F_{3,57} = 392.7$, $p < 0.0001$ for $\dot{V}_{O2}$, $F_{2,58} = 286.1$, $p < 0.0001$ for $\dot{V}_{CO2}$). There was no significant difference between metabolic rates across the three levels of hypoxia tested for any developmental stage, and all three developmental stages reached a similar minimum metabolic rate in severe hypoxia (3% $O_2$). Finally, although the degree of change was significantly different between each developmental stage (Fig. 2.1B&D), these differences were due to variance in normoxic metabolic rates.

To evaluate the role of thermoregulation in the hypoxic hypometabolic state across development we also measured $T_b$ (Fig. 2.1E&F). Subordinates, queens, and juveniles had a similar $T_b$ in normoxia, and animals at all three developmental stages exhibited a rapid decrease in $T_b$ with the onset of hypoxia ($F_{3,54} = 545.5$; $p < 0.0001$). The decrease in $T_b$ was most sensitive to hypoxia in queens, in whom $T_b$ decreased further in 7 and 5% $O_2$ than the other developmental stages. However, all three developmental stages had a similar magnitude of $T_b$ decrease in the most severe level of hypoxia tested (3% $O_2$).

We also calculated respiratory exchange ratios (RERs) to gain insight into fuel use in each condition and by animals in each developmental stage (Table 2.1). The RER is an indicator of fuel usage (e.g., carbohydrates or lipids): a value of 0.7 indicates lipid metabolism and a value of 1.0 indicates carbohydrate metabolism, whereas a value between 0.7 and 1 indicates a mix of both lipid and carbohydrate (154). With the onset of hypoxia, both subordinate and juvenile NMRs exhibited a shift from predominately lipids towards predominately carbohydrates ($F_{3,56} = 42.57$ $p < 0.0001$). Queens relied primarily on carbohydrates in all experimental conditions and did not exhibit an RER shift upon the switch to hypoxia. Notably, NMRs do not hyperventilate (through increased ventilation, the typical hypoxic ventilatory response (123, 155), and so this RER shift is not likely to be driven by a change in ventilation.

***Blood glucose is differentially elevated in hypoxia between developmental stages.*** We next evaluated the impact of acute hypoxic exposure on blood [glucose] (Fig. 2.2). In subordinate adults, queens, and juvenile NMRs, normoxic blood [glucose] was similar and blood [glucose] was only moderately or not significantly upregulated in 5 or 7% $O_2$. However, blood [glucose] increased ~ 2-fold above normoxic levels in all animals exposed to 3% $O_2$ for 1 h ($F_{1.4,33.59} = 121.5$; $p < 0.0001$). Conversely, the blood [glucose] of normoxic pups was significantly lower than in the other developmental stages, and exhibited a graded response to acute hypoxia exposure, such that blood [glucose] increased ~ 5-, 10-, and 15-fold in 7, 5, and 3% $O_2$, respectively, relative to normoxic levels ($p = 0.0017$, 0.0072, and 0.0005, respectively). The maximum blood [glucose] in pups was ~ 50% higher than that of the other developmental stages.

Subordinates and juveniles have a longer blood [glucose] latency than queens and pups. Next, we evaluated the ability of NMRs to re-establish blood glucose homeostasis after a bolus IP glucose challenge in normoxia or hypoxia (Fig. 2.3). For this comparison we utilized a hypoxia challenge of 3% $O_2$ because this was the level of hypoxia at which all age groups exhibited an increase in blood [glucose]. In normoxia, the peak magnitude of blood [glucose] following the glucose challenge was similar between all developmental stages (Fig. 2.3A&D; $F_{10,128} = 36.85$, $p < 0.0001$), but the time to peak blood [glucose] and the decay time representing glucose clearance rate were both greater in subordinates and juveniles (Fig 2.3A&C; $F_{3,13} = 8.73$, $p = 0.002$). Specifically, blood [glucose] reached a maximum value ~ 30-40 min post-injection in pups and queens versus ~ 60 min post-injection in juveniles and subordinate adults. Similarly, blood [glucose] returned to near baseline levels ~ 150 mins post-injection in pups and queens but it took between 180-240 mins for blood [glucose] to return to near baseline levels in juveniles and subordinate adults. Thus, the decay time from the peak in blood [glucose] to baseline was shorter for pups and queens than for subordinates and juveniles (Fig. 2.3A).

Intriguingly, hypoxia had only minor effects on peak blood [glucose], time to peak, or the time required to clear the exogenous glucose challenge in either queens or pups. Specifically, blood [glucose] reached a peak value ~ 30 min post-injection in queens and pups, and blood [glucose] returned to near baseline values after ~ 150 min (Fig. 2.3B-D; $F_{9,145} = 20.56$, $p < 0.0001$). Conversely, hypoxia considerably increased the peak magnitude of blood [glucose] and the clearance time in both subordinate adult and juvenile NMRs ($F_{3,25} = 16.24$, $p < 0.0001$), whereas the time to peak blood [glucose] was elevated in subordinates only. Specifically, blood [glucose] peaked and then plateaued between 60- and 120-min post-injection in these developmental stages, and at a peak magnitude that was ~ two-thirds greater than the blood [glucose] peak in animals at

the same developmental stage receiving a similar injection in normoxia. Furthermore, the time to clear this bolus glucose challenge was prolonged and blood [glucose] remained elevated above the normoxic glucose challenge peak for up to 5 h post-injection in subordinates ($F_{1,26}$ = 13.01, p = 0.0013), which was the limit permitted for experimentation under our animal ethics protocol. Thus, the decay time from peak blood [glucose] to baseline in hypoxia was considerably less for queens and pups than for subordinates and juveniles. We also analyzed the area under curve as a measure of total glucose mobilized to the blood by a given treatment. Here, values are consistently higher in hypoxia than normoxia across all groups, which suggests an increased glucose response when $O_2$ levels are lower. In contrast, there was no difference in total glucose mobilized in queens between normoxia and hypoxia, unlike the other groups. Subordinates had the largest amount of glucose mobilized in hypoxia, whereas the pups had the lowest amount in normoxia. Additionally, there were no significant differences in total glucose among any of the groups when in normoxia (Fig. 2.3E; $F_{3,38}$ = 65.1, p < 0.0001).

***Reoxygenation accelerates the rate of glucose clearance in subordinate NMRs.*** In a subset of experiments a group of subordinate adults were re-exposed to normoxia 1 h into the glucose tolerance test protocol in hypoxia (3% $O_2$; Fig. 2.4A; $F_{2, 105}$ = 47.95, p < 0.0001 for hypoxia). Upon reoxygenation, blood [glucose] rapidly began to decline and returned to pre-injection normoxic baseline in a similar timeframe as in animals undergoing a glucose tolerance test in normoxia. Calculation of total glucose supports these observations, indicating that the values during reoxygenation are close to those seen in normoxic condition (Fig. 2.4B $F_{2, 14}$ = 189.2, p < 0.0001).

***Queens and pups, but not adult subordinates, are insensitive to insulin in hypoxia.*** To elucidate the role of key hormones in the regulation of blood glucose, we next evaluated the sensitivity of NMR blood [glucose] to an insulin tolerance test (Fig. 2.5). In normoxia, insulin injection resulted in rapid decreases of blood [glucose] in queens and subordinate adult NMRs, such that blood [glucose] dropped significantly 30 min following insulin injection (Fig. 2.5A; $F_{8,40} = 10.5$; $p < 0.0001$). Conversely, insulin injection had no effect on blood [glucose] in pups in normoxia. In hypoxia, insulin similarly reduced blood [glucose] in adult subordinates and had no effect on pups; however, insulin injection also had no impact on blood [glucose] in hypoxic queens (Fig. 2.5B; $F_{4,24} = 4.38$, $p = 0.0084$), suggesting these animals were not sensitive to insulin in hypoxia. Analysis of total glucose indicates that insulin resistance increases under hypoxia compared to normoxia across all groups. Pups exposed to hypoxia display the highest insulin resistance, whereas subordinates and pups in normoxia show greater insulin sensitivity than the other groups (Fig. 2.5C; $F_{2,26} = 6.2$, $p = 0.0063$).

***Hypoxic blood [glucose] changes are IGF-1 sensitive in adults.*** Finally, we evaluated the impact of IGF-1 injection on blood [glucose] in adult subordinates and queens (Fig. 2.6). Here, IGF-1 tolerance tests during normoxia demonstrated that both queen and subordinate adult NMRs are sensitive to IGF-1 (Fig. 2.6A; $F_{7,21} = 264.1$, $p < 0.0001$). Similarly, in hypoxia, IGF-1 injection decreased blood [glucose] in both subordinate adults and queens (Fig. 2.6B; $F_{4,12} = 20.6$, $p < 0.0001$). Interestingly, the changes in blood glucose in normoxia were slower than in hypoxia. Specifically, the blood [glucose] of normoxic animals reached the lowest level at 120 ± 15 min post-IGF-1 injection, whereas during hypoxia, it was at 75 ± 15 min post-IGF-1 injection. Analysis of total glucose reveals significant differences between normoxic and hypoxic conditions across

all groups. Notably, under hypoxic conditions, total glucose mobilized in queens is higher than that for subordinates, suggesting that the queen group may experience a substantially greater overall blood glucose response compared to the subordinate group (Fig. 2.6C; $F_{1,12} = 1.74$, $p = 0.211$).

## 2.4 Figures and tables

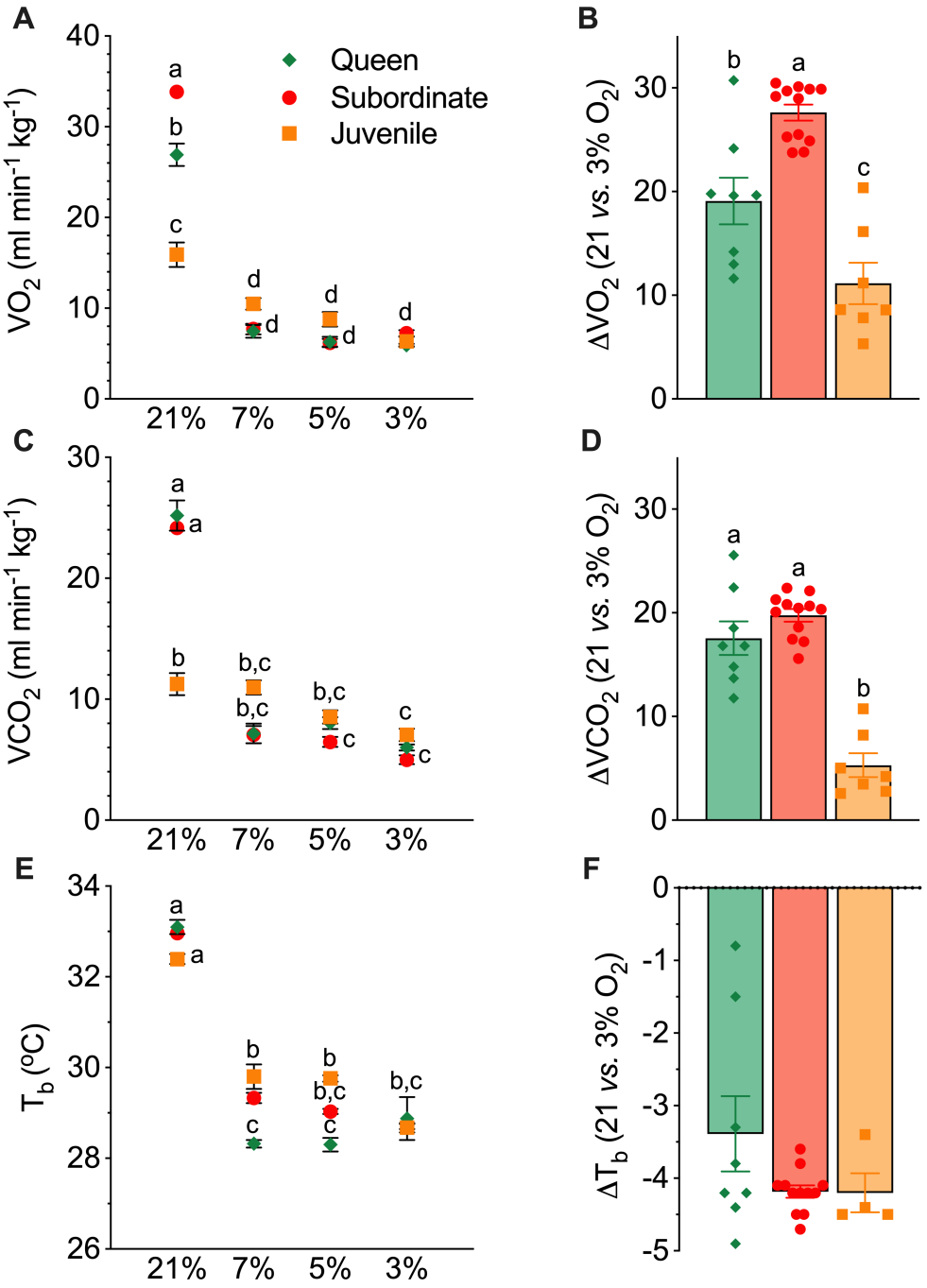


**Figure 2.1 NMRs decrease metabolic rate and body temperature in all hypoxic levels and developmental stages.** (A) $O_2$ consumption ($\dot{V}_{O2}$) from subordinate (n = 7-12), queen (n = 5- 8), and juvenile (n = 4-5) NMRs held for 1 h in normoxia (21% $O_2$), then exposed for 1 h in acute hypoxia (7, 5 or 3% $O_2$). (B) Change (Δ) in $\dot{V}_{O2}$ from animals treated in (A). (C) Carbon dioxide production ($\dot{V}_{CO2}$) from animals in (A). (D) $\Delta\dot{V}_{CO2}$ for data shown in (C). (E) Body temperature ($T_b$) for animals treated in (A). (F) $\Delta T_b$ for data shown in (E). Data are presented as mean ± SEM. Significant differences are indicated by different letters (Two-way ANOVA, $p < 0.05$).

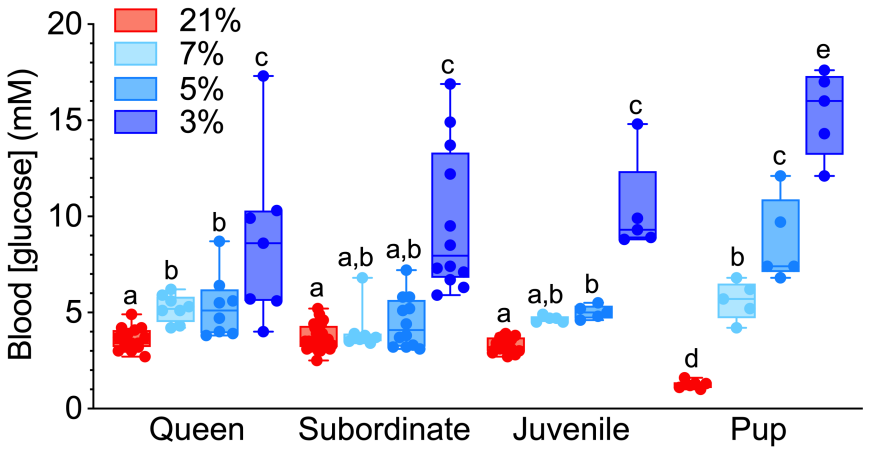


**Figure 2.2 Blood glucose increases with severe hypoxia in NMRs.** Each group of NMRs were exposed to 21% $O_2$ or hypoxia (3%, 5%, and 7%) for 1 h, as in Fig. S1. Data are presented as mean ± SEM. Significant differences are indicated by different letters (Two-way ANOVA ($p < 0.05$) for the pup group and two-way repeated measures ANOVA ($p < 0.05$) for other groups).

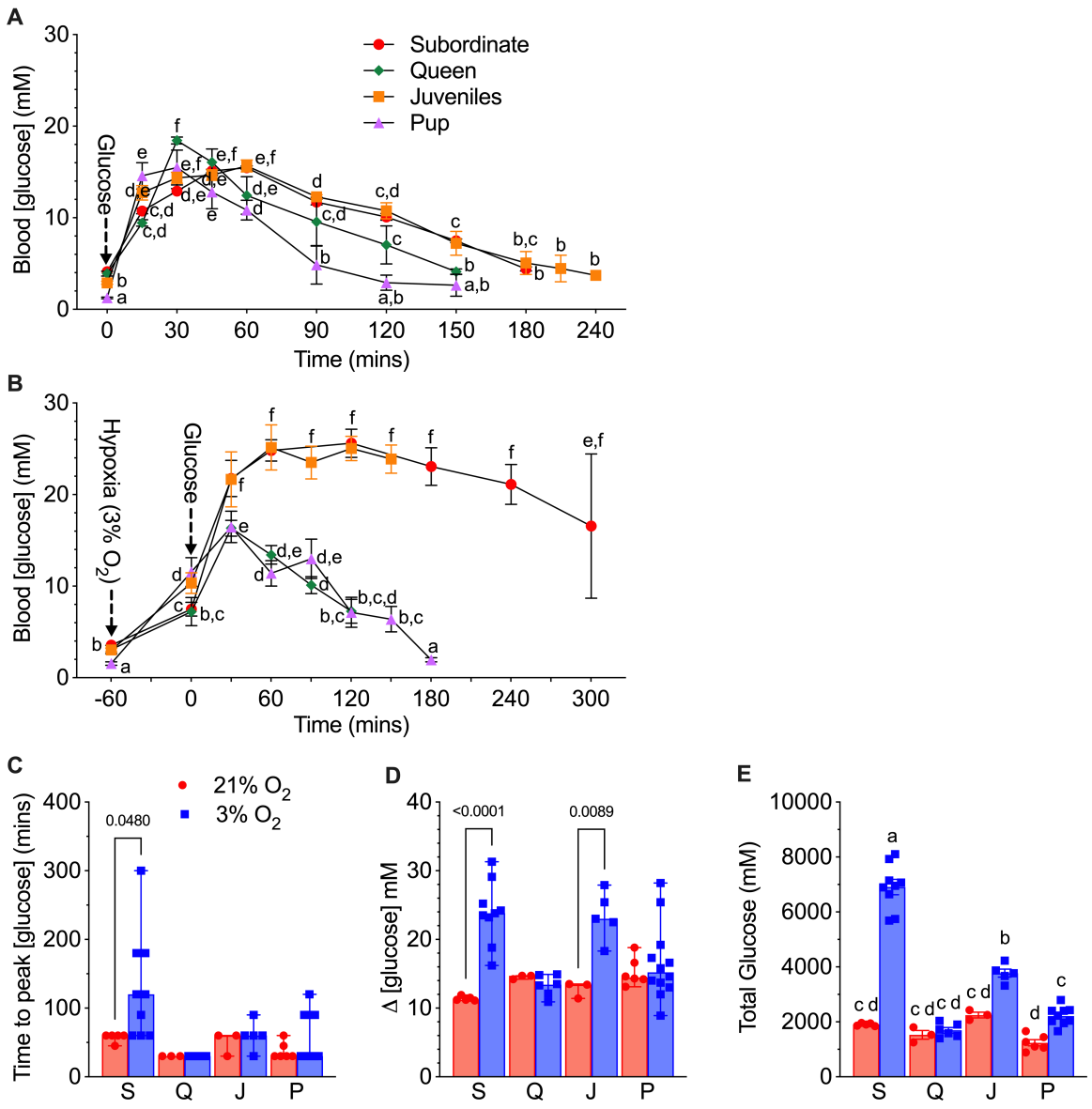


**Figure 2.3 Hypoxia increases the latency of glucose tolerance tests.** (A) Blood [glucose] before and after injection of 2 g glucose/kg body weight in normoxia (n = 5 for subordinates, 3 each for queens and juveniles, and 37 for pups). (B) Blood [glucose] before and after injection of 2 g glucose/kg body weight in hypoxia (3% $O_2$) (n = 9 for subordinates, 6 for queens, 5 for juveniles, and 51 for pups). (C) Time required to reach peak blood glucose value under normoxia and hypoxia. (D) Change (Δ) in blood glucose concentration from peak to baseline levels. (E) Total glucose (Area under the curve from the baseline level to the point of return to baseline value). Data are presented as means ± SEM. Arrows indicate onset of hypoxia exposure or point of glucose injection, as specified in each figure panel. Significant differences are indicated by different letters (Two-way ANOVA, $p < 0.05$ for the pup group and two-way repeated measures ANOVA, $p < 0.05$).

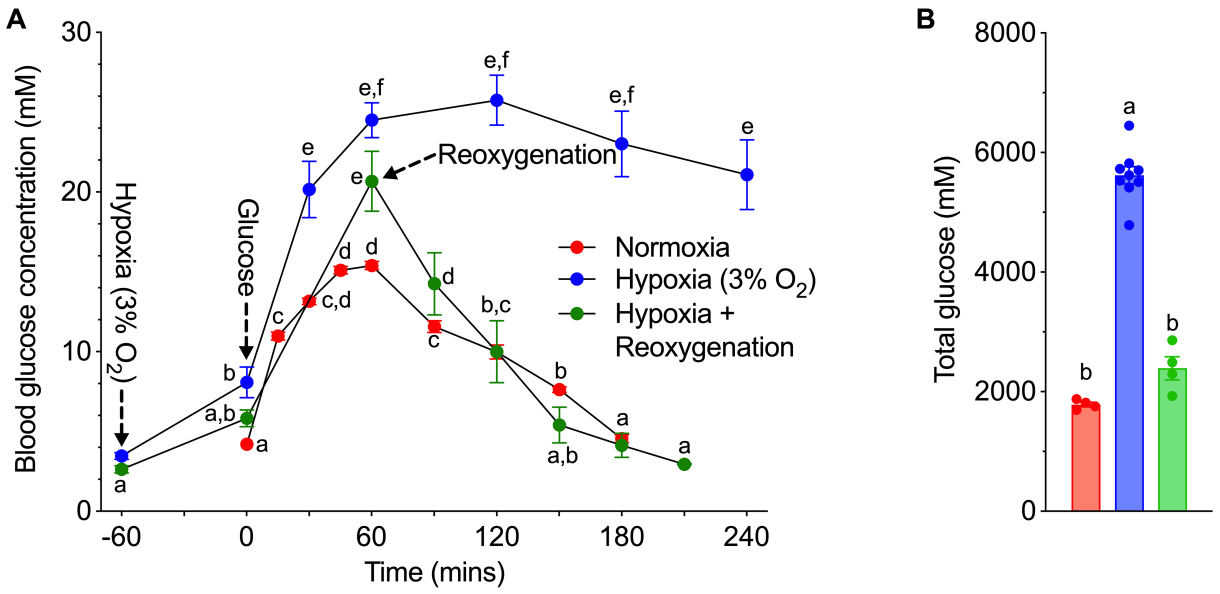


**Figure 2.4 Reoxygenation accelerates removal of glucose from the blood of subordinates NMRs.** (A) Blood [glucose] before and after injection of 2 g glucose/kg body weight in normoxia, hypoxia (3% $O_2$), or hypoxia followed by reoxygenation after 1 h (n = 4 each). (B) Total glucose (Area under the curve from the baseline level to the point of return to baseline value). Arrows indicate onset or offset of hypoxia exposure and/or point of glucose injection, as specified in the figure. Significant differences are indicated by different letters (One-way repeated measures ANOVA, $p < 0.05$).

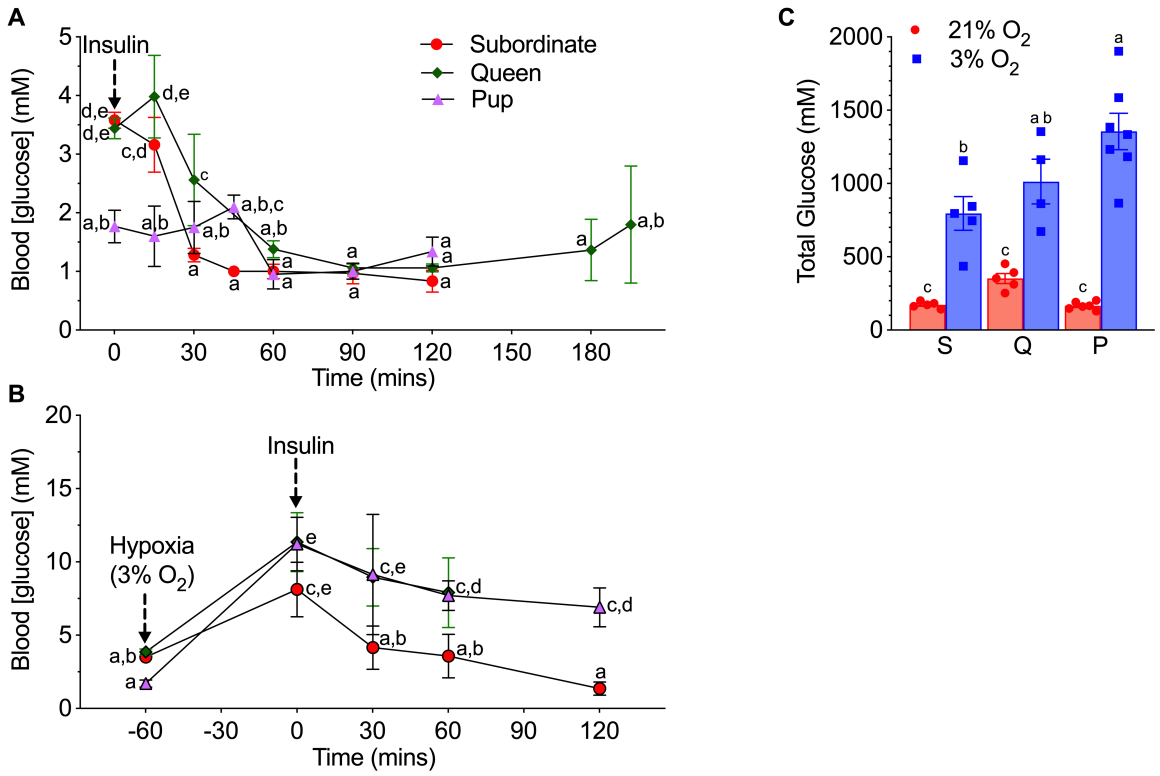


**Figure 2.5 Insulin reduces blood [glucose] in queens and subordinates but not pups.** (A) Blood [glucose] before and after injection of 0.75 U insulin/kg body weight in normoxia. (B) Blood [glucose] before and after injection of insulin in hypoxia (3% $O_2$). (C) Total glucose (Area under the curve from the baseline level to the point of return to baseline value). Data are presented as mean ± SEM (n = 5 for subordinates, 4-5 for queens, and 50 for pups). Arrows indicate onset of hypoxia exposure or point of insulin injection, as specified in the figure. Significant differences are indicated by different letters (Two-way ANOVA, $p < 0.05$ for pup group and two-way repeated measures ANOVA, $p < 0.05$ for other groups).

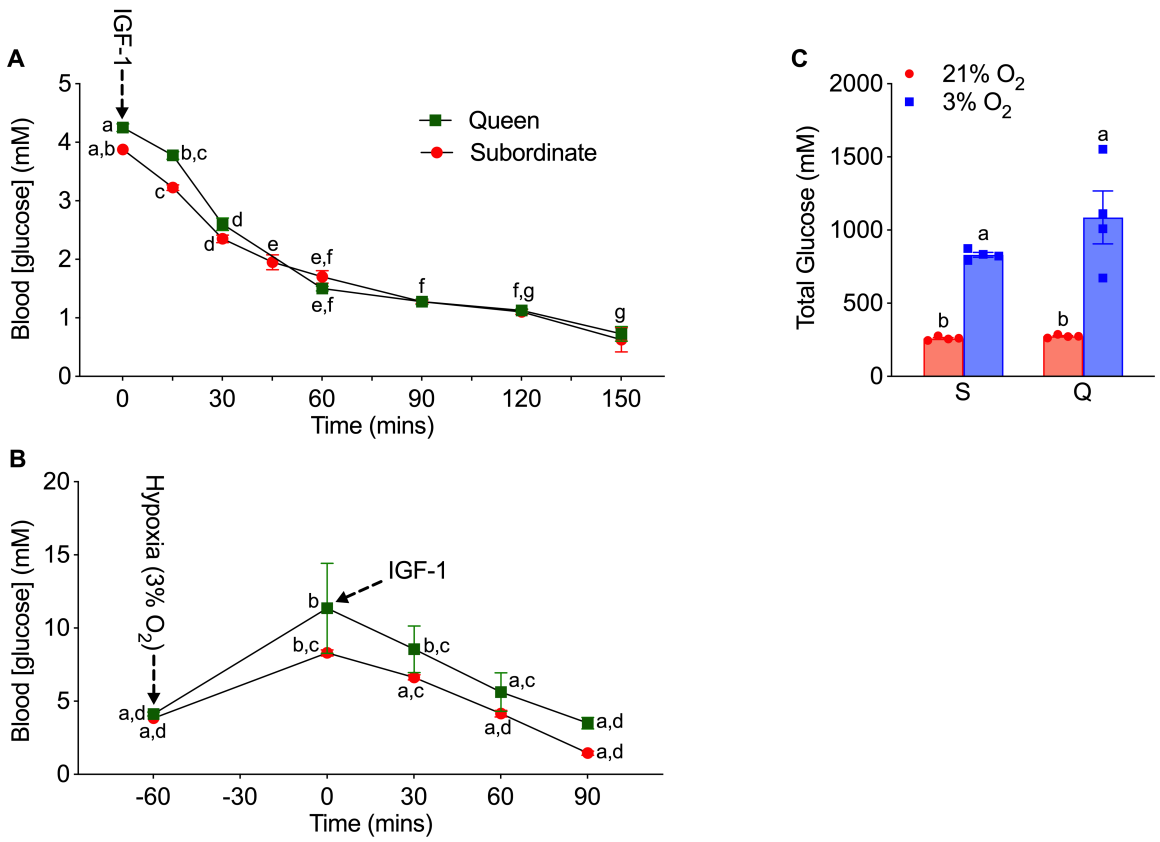


**Figure 2.6 IGF-1 reduces blood [glucose] in queens and subordinates.** (A) Blood [glucose] before and after injection of IGF-1 (1mg/kg body weight) in normoxia. (B) Blood [glucose] before and after injection of IGF-1 in hypoxia (3% $O_2$). (C) Total glucose (Area under the curve from the baseline level to the point of return to baseline value). Data are presented as means ± SEM from n = 4 each. Arrows indicate onset of hypoxia exposure or point of IGF-1 injection, as specified in the figure. Significant differences are indicated by different letters (Two-way repeated measures ANOVA, $p < 0.05$).

| Parameter | 21% $O_2$ | 7% $O_2$ | 5% $O_2$ | 3% $O_2$ |
|---|---|---|---|---|
| Queen | 0.95 ± 0.08 | 0.96 ± 0.06 | 1.02 ± 0.11 | 1.00 ± 0.11 |
| Subordinate | 0.71 ± 0.04$^{*\#}$ | 0.91 ± 0.12 | 1.05 ± 0.12 | 0.95 ± 0.08 |
| Juvenile | 0.72 ± 0.09$^{*\#}$ | 1.05 ± 0.03 | 0.99 ± 0.13 | 1.07 ± 0.09 |

**Table 2.1 Respiratory exchange ratios (RERs) in normoxia and hypoxia.** Summary of RER data from subordinate (n = 7-12), queen (n =5- 8), and juvenile (n = 4-7) NMRs held for 1 h in normoxia (21% $O_2$), then exposed for 1 h to acute hypoxia (7, 5 or 3% $O_2$). Data are presented as mean ± SEM. An asterisk (*) indicates significant differences between developmental stages within a given $O_2$ level, while pound ($^{\#}$) indicates significant differences between $O_2$ levels. Statistical significance determined by on-way ANOVA ($p < 0.05$) for pup group and one-way repeated measures ANOVA ($p < 0.05$) for other groups.

## 2.5 Discussion

Glucose is a key metabolic intermediate and is often released into the blood during hypoxia to support anaerobic metabolism. As such, differences in the regulation of blood glucose during hypoxia may be an important area of adaptation in hypoxia-tolerant species. In this chapter, we evaluated metabolism, $T_b$, and glucose handling during normoxia and hypoxia and across development in NMRs, which are among the most hypoxia-tolerant mammals (13, 23). Our study yielded several important findings. First, we describe metabolic and thermoregulatory responses to acute hypoxia across development for the first time in NMRs and report that animals of all developmental stages exhibit robust decreases in metabolic rate and thermogenesis in all levels of hypoxia tested. Conversely, blood glucose homeostasis is less sensitive to hypoxia than metabolism; juveniles and subordinates do not exhibit increased blood [glucose] until the most severe level of hypoxia tested whereas pups and queens exhibit increased blood [glucose] in moderate hypoxia and graded increases in blood [glucose] with progressively more severe hypoxia. Despite their high activation threshold for glucose mobilization by hypoxia, subordinates and juveniles have a higher blood [glucose] peak and take hours longer to clear a bolus glucose injection from the blood than pups or queens, suggesting that glucose handling is impaired by hypoxia in this species. Remarkably, glucose clearance accelerates to match the normoxic clearance rate if subordinate animals are returned to normoxia, confirming that the cause of this impairment is $O_2$-sensitive, at least at this developmental stage. Presumably this impairment is at the level of insulin release since exogenous insulin induces the removal of glucose from the blood in all developmental stages and experimental conditions, indicating that the insulin receptor remains functional. Conversely, queens and pups have reduced sensitivity to insulin during hypoxia, suggesting that insulin receptor function is modulated by hypoxia in sexually-developed

females. However, these animals remain sensitive to IGF-1, which presumably allows them to maintain active regulation of blood glucose in hypoxia. Together, these results indicate that 1) glucose mobilization is not a primary response to reduced metabolic demand during low $O_2$ availability and is only recruited in severe hypoxia in most NMRs, and 2) the signalling pathways that regulate glucose are variably modified by hypoxia, depending on developmental and/or reproductive stage.

***NMRs of all developmental stages reduce metabolic demand and thermogenesis in hypoxia.*** Previous studies overwhelmingly agree that, in response to acute hypoxia exposure, subordinate adult NMRs exhibit a robust hypoxic metabolic response characterized by a decrease in metabolic rate of up to 85%, a cessation of non-shivering thermogenesis that allows $T_b$ to drop to near ambient temperatures, and a fuel substrate switch from mixed lipids/carbohydrates to entirely carbohydrates (23, 121, 123-125, 144, 148, 149, 156-159). In the present study we extend these findings to juveniles and breeding female NMRs (queens) and demonstrate that, like adult subordinates, these developmental groups also reduce $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ progressively with increasingly deeper levels of hypoxia, reduce $T_b$ to near ambient levels, and rely on carbohydrate metabolism in hypoxia. Conversely, most other small mammals, including rats, mice, hamsters, and ground squirrels utilize divergent physiological strategies for coping with hypoxia depending on their stage of development (160-163). For example, most other adult rodents exhibit a lesser depression in metabolic rate under hypoxic conditions compared with neonates or juveniles of the same species, and this correlates with diminished hypoxia-tolerance in adults relative to neonates.

Regardless of developmental stage, a switch in primary metabolic fuel substrate usage from lipid to carbohydrates during hypoxia provides a greater yield of ATP per mole of $O_2$ consumed,

which can increase energy efficiency, and is often observed in small hypoxia-tolerant mammals (110, 164). In NMRs, this shift is supported by the mobilization of glucose stores from the liver and an increase in blood glucose levels during severe (123), but not moderate hypoxia (current study, see below). Combined with a significant suppression of metabolic rate, this mobilization may help to limit the need for anaerobic metabolism and reduce the subsequent $O_2$ debt that occurs after severe hypoxia.

***Adult and juvenile NMRs mobilize glucose only in severe hypoxia.*** In general, most hypoxia-intolerant species have variable glucose responses to hypoxia, which are often characterized by reductions in blood [glucose], whereas hypoxia-tolerant species exhibit elevated blood [glucose] in hypoxia or anoxia (see introduction and (66, 71, 111, 165, 166). In good agreement with the pattern presented by these studies, we report increased blood glucose during hypoxia in all developmental stages of NMRs. This response is most sensitive in queens and pups, which exhibit increased blood [glucose] in 7% $O_2$. Conversely, juveniles and subordinates do not exhibit increases until 5 and 3% $O_2$, respectively. Where present, changes in blood glucose induced by 7 and 5% $O_2$ are mild but then jump markedly in 3% $O_2$. Taken together, these data demonstrate that NMRs at most developmental stages require a very strong hypoxic stimulus to mobilize glucose into the blood. This is important because elevations of glucose in blood may occur due to a release of stored glucose from tissue (primarily liver) into the blood and/or a reduction in glucose uptake by metabolizing tissues themselves, such as occurs during periods of metabolic rate suppression. Importantly, the suppression of metabolic rate that we observe in all developmental stages in 7% $O_2$ is near the maximum suppression observed in deeper levels of hypoxia, but 7% $O_2$ is a level of hypoxia that is well above the threshold required for significant glucose accumulation in the blood

of these animals. Therefore, the hypoxic increase in blood [glucose] is likely not mediated by a decrease in tissue uptake. In support of this, we have previously demonstrated that the expression of GLUT-4 (which is the primary mediator of glucose uptake into cells), and phosphorylated AMPK (which activates glucose uptake and glycolysis) are downregulated in total NMR skeletal muscle during moderate hypoxia (7% $O_2$ for 4 hr; (167)), which would contribute to reduced glucose uptake by that tissue. However, these measures reflect only total protein level and not the functional activity of GLUT4 or AMPK. In the case of GLUT4, glucose uptake is regulated not by de novo synthesis but by the translocation of pre-existing vesicles to the plasma membrane in response to insulin or AMPK signaling (168). Thus, our measurements capture only a limited intracellular pool of GLUT4, and do not account for trafficking defects or impaired activation. Future studies should include membrane localization assays or post-translational modification analyses to fully clarify these regulatory effects. Conversely, in 3% $O_2$, blood [glucose] increases sharply in all developmental stages and liver [glucose] plummets (123), suggesting that glucose stores are mobilized from the liver only in severe hypoxia, presumably to support increased anaerobic activity in tissues. Testing GLUT4 and AMPK activation under these more extreme conditions would help determine whether their regulation is $O_2$-level dependent and whether tissue-specific responses differ across castes and organs.

***Divergent regulation of blood [glucose] in hypoxia.*** Glucose tolerance tests indicate that NMRs are glucose tolerant across all developmental stages in normoxia (119). Conversely, we report starkly different responses to glucose injection in hypoxia across development, such that queens and pups retain glucose tolerance in hypoxia, whereas juveniles and subordinate adults are less able to effectively clear the exogenous glucose bolus from their blood, suggesting that the

signalling mechanisms responsible for mediating this removal are impaired in hypoxia. These differences are clearly $O_2$-sensitive because when subordinate adult NMRs are reoxygenated following 1 h of hypoxia post-injection, blood [glucose] rapidly declined to baseline values.

Insulin is the primary initiator of glucose uptake in most mammals and insulin signaling is upregulated by hypoxia in hypoxia-tolerant newborn calves (66) and juvenile and newborn rats (166), but decreases in hypoxia-intolerant adult rats (169). Unfortunately, we were unable to measure insulin levels in NMRs because commercial insulin kits do not effectively detect insulin in this species (126), presumably because NMRs (along with other hystricognaths) have mutations in their insulin ß-chain sequence (140), which are consistent with reduced affinity for insulin receptors (170, 171). Nonetheless, and consistent with a previous study in NMRs (126), insulin tolerance tests in normoxia demonstrate that NMRs are responsive to insulin. Furthermore, similar tests in hypoxia indicate that subordinates but not queens and pups remain sensitive to insulin when $O_2$ is limited. These findings raise the possibility that the insulin β-chain mutation in NMRs may reflect an adaptive modification rather than a dysfunctional one. Although this mutation likely reduces binding affinity, our results using insulin suggest that insulin receptors might remain functional. Whether native NMR insulin is less effective or simply co-evolved with altered receptor dynamics remains unknown. Future studies using purified NMR insulin could clarify whether this change represents a functional impairment or an evolved strategy for regulating glucose under hypoxia. These findings point to a developmental and caste-specific regulation of insulin signaling under hypoxia. While insulin tolerance tests indicate that subordinate adults retain insulin responsiveness under low $O_2$, they nonetheless fail to clear exogenous glucose, suggesting that glucose sensing or insulin release, rather than receptor function, may be impaired. In contrast,

queens and pups show blunted insulin responses in hypoxia, pointing to a possible disruption in insulin receptor signaling specific to reproductive and early-life stages.

Sex hormones have marked impacts on insulin signalling; therefore, it is possible that queens develop a response that is different from subordinate animals as they undergo sexual differentiation. For example, estrogen plays an important role in regulating insulin sensitivity and the expression of gluconeogenesis enzymes (172, 173). Specifically, estrogen indirectly inhibits Foxo1, which is a regulator transcription factor for hepatic glucose production and promotes the gene expression of gluconeogenesis enzymes via activation of PI3K-AKT signaling (174, 175). Estrogen levels are higher in queens than subordinates NMRs (176), which may explain some of these developmental effects. Furthermore, the hormonal control of glucose regulation in pups may be like in queens if pups ingest estrogen from the mother while nursing. Then, as animals are weaned and develop into sexually supressed subordinate adults, their estrogen levels would fall concomitant with changing insulin sensitivity. Further study is needed to investigate how estrogen may impact blood glucose and insulin signaling in this species.

It is also notable that in most mammals the insulin signalling system is not functional until post-birth; instead, insulin-like growth factors regulate blood [glucose] through insulin receptors at this developmental stage (177). Post-birth, the IGF system declines, and insulin signalling takes over in most mammals (178). The IGF system of NMRs is functionally similar to that of humans and mice (179), indicating that there may not be significant adaptations in this signaling pathway in NMRs per se; however, the expression of IGF and its receptors persists into adulthood in NMRs (140), unlike in most other mammals. Together with the mutations to the insulin sequence discussed above, these data suggest that, unlike most adult mammals, NMRs may retain a fetal IGF signalling pathway into adulthood. Of particular interest is IGF-1, which indirectly enhances

glucose uptake by promoting the expression of glucose transporters, such as GLUT 4 (180). IGF-1 can also promote the activity of enzymes involved in glucose metabolism, such as hexokinase and pyruvate dehydrogenase, thereby enhancing the utilization of glucose for ATP production in cells (181). We found that both queen and subordinate NMRs are sensitive to IGF-1 injection during both normoxia and hypoxia, suggesting that IGF signalling indeed plays a crucial role in regulating glucose metabolism in NMRs and may therefore provide queens with a mechanism to regulate blood [glucose] in hypoxia when they are insensitive to insulin signalling.

***Study limitations.*** A key limitation of our study is the absence of a sham control group; repeated animal handling the administration of injections, and the collection of blood without the use of anesthetic may have induced stress. Such stress could lead to elevations of catecholamines and glucocorticoids, which can in turn affect blood glucose levels. In this study, our objective was to compare changes across different $O_2$ level groups. Consequently, we ensured that all groups were subjected to identical handling procedures if possible and thereby standardizing the stress from handling across most experimental conditions (excluding pups, which were sampled a single time each). We believe that this design choice, along with our consideration of the physiological effects of stress on blood glucose levels, supports a valid comparison of the impact of hypoxia on blood glucose across development. However, this caveat should be kept in mind when evaluating our results.

***Conclusions.*** Our study provides insight into the importance of glucose in the reorganization of NMR metabolism during hypoxia, and the function of insulin and IGF-1 signaling in this regulation. An intriguing conclusion that may be drawn when considering the ecophysiological

significance of our results is that NMRs likely do not experience the severe depths of hypoxia in their natural habitats that are required to induce glucose mobilization in our study (13, 23, 155, 182). This is important because it suggests that NMRs do not need to release glucose stores into the blood to support anaerobic metabolism except when approaching the limits of their hypoxia tolerance (123, 144). Presumably, this indicates that NMRs can effectively and coordinately down-regulate energy demand to match declining energy supplies in moderate hypoxia at all stages of development. Furthermore, queens, as the reproductive and dominant individuals in the colony, appear to have evolved a strategy to regulate their blood [glucose] efficiently, even in severe hypoxia, when insulin-mediated glucose regulation appears to be otherwise disabled in adult NMRs. NMRs putatively experience hypoxia primarily in their nest chambers and queens remain in these chambers far longer than other conspecifics while birthing, feeding, and caring for pups (13, 23), which would also require significant energy. Therefore, queens likely have the most regular and intense exposure to hypoxia in the colony but also have the highest metabolic demands due to reproductive costs. As such, reproductive females may require anaerobic energy production more frequently or in less severe levels of hypoxia than do subordinate adults or juveniles and thus need to retain the ability to recruit glucose reserves to support tissue-level carbohydrate metabolism during periods of hypometabolism in hypoxia. Alternatively, queens may need to reserve lipids to produce milk or as building blocks for growing fetuses and thus require a more ready and rapid pool of carbohydrate substrate in all conditions. In either case, this framework is supported by our observation that blood [glucose] becomes elevated in queens at a less severe level of hypoxia than in subordinate animals. Further research into the mechanisms underlying IGF-1 regulation and potential interactions between estrogen and glucose homeostasis in NMRs is clearly

warranted to better understand the metabolic adaptations of this species and their potential relevance to human health.

# Chapter 3: Severe acute hypoxia upregulates anaerobic metabolism in non-reproductive but not queen naked mole-rats

This chapter includes material from the following article which has been accepted by the Journal of Experimental Biology:

Ojaghi, M., & Pamenter, M.E. (2025). Severe acute hypoxia upregulates anaerobic metabolism in non-reproductive but not queen naked mole-rats.

## 3.1 Introduction

Unlike many hypoxia-tolerant species that enter torpor-like states during severe hypoxia to conserve energy (104, 183), NMRs remain active by significantly reducing their metabolic rate (125, 148, 159). Remarkably, NMRs achieve this degree of metabolic rate suppression without developing significant metabolic acidosis or $O_2$ debt (123). This resilience may be due to their unique capacity to switch metabolic substrates, enhance the efficiency of energy production, and/or utilize buffering mechanisms that mask typical signs of metabolic acidosis.

Studies across various organs suggest that NMRs employ a tissue-specific suite of metabolic responses to hypoxia. For example, in brain there is significant metabolic suppression (156, 158, 184), with a shift towards the pentose phosphate pathway to maintain redox balance and produce NADPH, which protects against oxidative stress (185). In brown adipose tissue, non-shivering thermogenesis ceases, helping NMRs lower their body temperature to conserve energy (124). In skeletal muscle, mitochondrial function is only mildly reduced by hypoxia (186), but AMPK (5′-AMP-activated protein kinase) activity is downregulated (167). This downregulation, associated with decreased expression of key AMPK subunits such as AMPK-α2 and phosphorylated AMPK (p-AMPK), leads to reduced expression of proteins involved in glucose metabolism, such as GLUT4 and phosphorylated glycogen synthase, consistent with reduced metabolic flux through skeletal muscle. Conversely, cardiac muscle may increase metabolic demands in hypoxia and has large glycogen stores, which are crucial during hypoxic conditions. These glycogen reserves can be utilized to produce ATP, ensuring that the heart continues to function effectively even in hypoxia (187). Indeed, the NMR heart maintains or even increases its metabolic activity during hypoxia relative to systemic energy demands by sustaining the activity of key enzymes involved in glycolysis, the TCA cycle, and fatty acid oxidation (158).

Our findings in chapter 2 showed that NMR blood glucose levels increase during severe hypoxia and whole-animal fuel substrate use shifts from lipids to carbohydrates, possibly due to a net reduction of glucose uptake by tissues and/or increased glucose release into the bloodstream. Indeed, glucose homeostasis in the NMR liver is altered in hypoxia, possibly conserving energy by reducing glucose release in moderate hypoxia (185), but then releasing glucose to the blood during severe hypoxia, as indicated by a decrease in liver glucose levels alongside a concurrent increase in blood glucose at this level of exposure (123, 188).

Despite these advances, almost all that is known about the NMR responses to hypoxia comes from studies in subordinate (non-reproductive) adult animals and little to nothing is known about how juvenile or reproductive animals respond to hypoxia. To address this, we asked to what degree anaerobic metabolic pathways are activated during severe hypoxia across major tissues and developmental stages in NMRs. We measured blood lactate, glycolytic enzyme activity, and the expression of enzymes involved in gluconeogenesis and glycogen synthesis/breakdown, and lactate transport, from animals held in normoxia or severe hypoxia (3% $O_2$) for 1 hr.

We hypothesized that robust metabolic rate suppression in hypoxic NMRs reduces their reliance on anaerobic pathways by minimizing overall energy demands. This adaptation likely works in tandem with other mechanisms to support hypoxia tolerance, such as selective and tissue-specific up-regulation of anaerobic metabolism or increased efficiency of $O_2$ utilization. We predicted that 1) glycolytic enzyme activity would vary by tissue: activity in tissues with high obligatory metabolic activity, such as brain, heart, and liver, would increase to support neuroprotection, meet cardiac energy demands, and mobilize glucose for systemic energy support, respectively, whereas glycolytic enzyme activity in muscle and kidney would decrease to conserve energy in these less critical tissues, 2) enzymes involved in glycogen metabolism and

gluconeogenesis, which are $O_2$-dependent processes, would be downregulated in non-essential tissues such as muscle and kidney to conserve energy, but maintained or upregulated in essential tissues like the liver to support systemic glucose production, and 3) MCT4 expression would be downregulated, leading to an accumulation of lactate in the blood, along with increased glycolysis in some tissues to meet energy demands. Considering developmental stages, we predicted that juvenile and subordinate NMRs would display a greater reliance on anaerobic pathways than queens, reflecting their roles in the colony as workers requiring more energy to perform tasks such as foraging and burrowing, whereas queens tend to be less active in the colony and spend more time resting in the nest. To test these predictions, we exposed juvenile, adult subordinate, and queen NMRs to 1 hr of normoxia (21% $O_2$) or hypoxia (3% $O_2$) and collected tissues for molecular analysis.

## 3.2 Materials and methodology

### 3.2.1 Animals and experimental design

A total of 24 NMRs were included in this study, including 8 adult subordinates (1–13 years old; 55 ± 10 g), 8 queens with previous successful litters but not currently pregnant or lactating (3–12 years old; 65 ± 8 g), and 8 juveniles (~ 2 months old; 13 ± 2 g). Animals were equally and randomly divided between a normoxic control group and a hypoxic (3% $O_2$ for 1 hr) treatment group. After exposure, animals were quickly euthanized by cervical dislocation, following by immediate decapitation. Animals were handled gently to minimize stress prior to decapitation as stress-induced muscle contraction could contribute to lactate production and were decapitated within 10 second of removal from the treatment chamber. Anesthesia was not used to avoid introducing confounding effects on blood and tissue lactate levels. Blood was collected for lactate measurements and tissues, including the brain, heart, liver, muscles, and kidney, were sampled within 2 min over ice, immediately frozen in liquid nitrogen, and stored at −80°C until molecular analysis.

### 3.2.2 Blood lactate

Approximately 30 μl of blood was used to measure lactate levels with a StatStrip Xpress Lactate Analyzer (Nova Biomedical, Waltham, MA, USA).

### 3.2.3 Measurement of gene expression by qPCR

Total RNA was extracted from 50–100 mg of frozen, powdered tissue using TRIzol LS reagent (Thermofisher Scientific) according to the manufacturer's instructions. RNA concentrations and purities were determined using a NanoDrop ND-1000 Spectrophotometer, and the RNA was stored

at −80°C. Following genomic DNA removal, cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. The cDNA was then stored at −20°C.

qPCR was conducted using Maxima SYBR Green qPCR Master Mix (Bio-Rad) on a Rotor-Gene Q real-time PCR machine (Qiagen). Reactions were performed in a total volume of 10 µl according to the manufacturer's instructions. Primer sequences used in this study are listed in Table. 3.1. Two reference genes, eukaryotic elongation factor 2 (EEF2) and Actb (beta-actin), were used in this study. These genes were selected based on their stable expression across all experimental conditions and tissues, as validated in a previous study (189), and confirmed in our study. Each sample was run in duplicate, accompanied by negative controls, including a no-template control (where cDNA was replaced with water) and a no-RT control (where the cDNA synthesis reaction was carried out without reverse transcriptase). The cycling conditions were as follows: 30 seconds at 95°C, followed by 40 cycles of 10 seconds at 95°C and 25 seconds at 60°C. Melt curve analysis was conducted as per the manufacturer's protocol. Standard curves were generated using serially diluted pooled samples, and relative transcript abundances were calculated using the Pfaffl method (190).

### 3.2.4 Enzyme assays

Enzyme activities were determined using a Spectra Max Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA). To assess the activities of the key glycolytic enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH), 50 mg of frozen tissue samples were weighed and homogenized on ice in 19 volumes of extraction buffer [25 mM Tris-HCl, 1 mM EDTA, with 5 mM dithiothreitol (DTT) and 0.05% (vol/vol) Triton X-100 added fresh on the day

of the experiment]. The homogenates were centrifuged at 2,400 g for 5 min at 4°C, and the supernatant was stored at −80°C until further analysis. Assay conditions were optimized using skeletal muscle to achieve maximal enzyme activity, which may not represent the maximal rate in all tissue types. Homogenates underwent a freeze/thaw cycle, and preliminary tests confirmed that substrate and cofactor concentrations were saturating but not inhibitory. Control reactions, lacking substrate, were run in parallel for each enzyme to account for any background activity. All assays were conducted in triplicate at the NMR body temperature of 32°C under the following conditions (158):

PK: [A340; pH 7.35] (191): 0.17 mM NADH, 5 mM ADP, 80 mM KCl, 10 mM MgCl2, and 5 mM phosphoenolpyruvate (PEP) (omitted from the control), with excess coupling enzyme (LDH) in 160 mM triethanolamine/HCl.

LDH: [A340; pH 7.3] (192): 0.17 mM NADH, 1 mM KCN, and 2 mM pyruvate (omitted from the control) in 50 mM Tris-HCl.

### 3.2.5 Statistical analysis

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Significant differences ($P<0.05$) were determined using one-way and two-way ANOVA to assess intraspecific differences between normoxia and hypoxia (3% $O_2$) for each independent variable. When a significant interaction or main effect was identified, Šidák's multiple comparisons test was applied to determine specific differences between normoxia and hypoxia within each treatment group. Data are expressed as mean ± s.e.m., with asterisks or letters marking significant differences as indicated in the figure legends.

### 3.3 Results

***Severe acute hypoxia increases blood lactate in all developmental stages***. Analysis with a two-way ANOVA revealed a significant effect of hypoxia on blood lactate levels across all developmental stages but no differences between baseline lactate levels across developmental stages (Fig. 3.1A, n = 4 for each group and treatment, $F_{1,18} = 337.8$, $P < 0.0001$; effect of hypoxia on blood lactate). Specifically, in normoxia, subordinates and juveniles exhibited relatively low blood lactate levels (~ 1.2 mM), whereas queens had higher levels (~ 3 mM); however, this difference between groups was not significant (P = 0.3635 vs. juveniles and 0.4710 vs. subordinates). As a result, the maximum blood lactate concentration in queens during hypoxia was ~ 30% higher than that of the other developmental stages ($F_{2,18} = 10.92$, P = 0.0008 for the effect of developmental stage groups on blood lactate). However, analysis of the magnitude of change across ontogeny indicated that there was no difference in the net increase of blood lactate between groups (Fig. 3.1B; $F_{2,9} = 1.524$, P = 0.2691).

***Severe acute hypoxia alters MCT4 expression differently across developmental stages***. To determine which tissues might contribute to elevated blood lactate, we measured the expression of the MCT4 gene across various tissues in all groups under normoxic and hypoxic conditions (Fig. 3.2, n = 4 for each tissue and treatment). MCT4 facilities the export of lactate from cells, particularly in glycolytic tissues, preventing acid buildup during anaerobic metabolism (193). MCT4 expression is sensitive to hypoxia and is upregulated during hypoxic conditions (47). In normoxia, MCT4 expression was highest in brain across all developmental stages (Fig. 3.2A) and was 2-3 fold lower in all other tissues (Fig. 3.2B-E). In addition, baseline MCT4 expression was higher in queens than in juvenile or subordinate adults in normoxic muscle, kidney, and heart

tissues. With hypoxic exposure, MCT4 expression decreased by ~ 90% in subordinate and juvenile NMR brain, but was not significantly reduced in queens, remaining ~ 5-fold higher than in other developmental stages treated with hypoxia. In muscle, heart, and kidney, MCT4 expression tended to increase with hypoxia in juvenile and adult subordinates, but this did not reach significance in all cases (Fig. 3.2B-D). In hypoxic queens, MCT4 expression was decreased (muscle) or unchanged (heart and kidney). Liver MCT4 expression was not impacted by hypoxia in any developmental stage (Fig. 3.2E).

***Divergent glycolytic enzyme changes across developmental stages during severe acute hypoxia***. Next, we analyzed enzymatic activity of the two key glycolytic enzymes LDH and PK. This analysis revealed distinct variations in activity across tissues of subordinate and juvenile animals compared to queens (Figs. 3.3&4, Tables 3.3&3.4; $n$ = 4 for each tissue and treatment). In normoxia, baseline LDH and PK activity was high in muscle and liver and low in kidney and heart in all developmental stages. Interestingly, baseline LDH and PK activity in brain diverged, with PK activity being highest in brain in all developmental stages but LDH activity being lowest in brain in all developmental stages. Finally, normoxic LDH and PK enzyme activities were higher in queens than subordinate adults or juveniles in all organs examined.

In hypoxia, enzyme activity tended to increase or be unchanged in non-breeding subordinate juveniles and adults but tended to decrease or be unchanged in queens. Specifically, LDH activity did not change following hypoxic exposure in the heart and liver of subordinates and juveniles but increased ~ 30% in muscle and ~ 50% in the kidney. Brain tissue was an outlier to this pattern, with LDH activity decreasing ~ 33% (Fig. 3.3A-E). A different phenotype was observed in queens, in which there was no change in LDH activity in hypoxic brain, heart, liver or

kidney, but a 32% decrease in muscle activity during hypoxia (Fig. 3.3A-E). A similar pattern between developmental stage and enzyme activity was observed for PK. There were no changes in PK activity in the brain and liver of subordinates or juveniles. However, PK activity increased ~ 20% in the muscle of subordinates, ~ 45% in the muscle of juveniles, ~ 35% in the heart of subordinate adults and juveniles, and ~ 40% in the kidney of both groups (Fig. 3.4A-E). Conversely in queens, there was no hypoxia-mediated change in PK activity in the brain, heart, liver, or kidney, but there was an ~ 30% decrease in muscle (Fig. 3.4A-E).

***Tissue-specific regulation of glycogen metabolism in NMRs under severe acute hypoxia***. Next, we measured the gene expression of enzymes involved in glycogen synthesis and breakdown to understand how glycogen metabolism changes across different tissues and developmental stages under hypoxic condition. Specifically, we examined glycogen synthase 2 (GYS2) as an indicator of glycogen synthesis in liver; phosphorylase kinase gamma 1 (PHKG1) and glycogen phosphorylase (PYGL) as indicators of glycogen breakdown in liver; glycogen phosphorylase (PYGM) and phosphorylase kinase gamma 2 (PHKG2) as indicators of glycogen breakdown in muscle; and glycogen phosphorylase (PYGB) as an indicator of glycogen breakdown in brain (Fig. 3.5-7; n = 4 for each tissue and treatment). This tissue-specific distinction is based on the well-documented roles of these isoforms in glycogen metabolism, as supported by previous studies (194, 195).

In normoxic liver, the expression of all three genes was similar across developmental stages/castes, although PYGL was slightly higher in queens than in juvenile or subordinate adult animals (Fig. 3.5A-C & Table 3.5). Conversely, with hypoxia exposure the expression of PHKG1 increased by ~ 3-fold in the liver of subordinates and juveniles but was unchanged in queens.

GYS2 and PYGL remained unchanged during hypoxia across all groups. Notably, GYS2 expression decreased by ~ 50% in both juveniles and adult subordinates, but this change did not reach statistical significance, likely due to a low sample size.

In normoxic muscle, PHKG2 and PYGM expression were almost undetectable in juvenile and adult subordinates but were comparatively quite high in queens (Fig. 3.6A-B & Table 3.5). With hypoxia exposure, the expression of PHKG2 and PYGM was unchanged in juveniles and adult subordinates, whereas the expression of both enzyme genes decreased in queens (by ~ 50% for PHKG2 and by ~ 70% for PYGM).

Next, we measured PYGB gene expression in brain to understand how glycogen breakdown contributes to energy production during hypoxia, particularly given that metabolic rate suppression in the hypoxic NMR brain (156, 158, 184) is accompanied by a switch to the pentose phosphate pathway (185). We did not measure PYGB gene expression in other tissues because glucose is the main substrate for energy production in the brain, whereas other tissues may rely on alternative substrates and metabolic pathways during hypoxia (196). We found that NMR brain PYGB gene expression decreased by ~ 50% during hypoxia across all developmental stages (Fig. 3.7 & Table 3.5, n = 4 for each group and treatment); however, this change was statistically significant only in the juvenile and subordinate groups.

***Opposing regulation of gluconeogenesis in queens vs. subordinates and juveniles***. Finally, we analyzed the expression of gluconeogenesis enzymes in the liver and kidney of subordinate, juvenile, and queen NMRs under normoxic and hypoxic conditions to determine whether NMRs utilize blood lactate more for glucose production during hypoxia than normoxia, thereby potentially supporting the glycolysis pathway and preventing blood acidosis (Fig. 3.8, n = 4 for

each tissue and treatment). We found that the expression of both genes was higher in queen liver than in subordinate adult or juvenile liver, but no differences across caste was observed in kidney (Fig. 3.8A-D & Table 3.6). Acute hypoxia increased G6Pase expression in the liver by ~ 80% in subordinates and 60% in juveniles, while it remained unchanged in the kidney. Additionally, the expression of PCK remained unchanged in both tissues of juveniles and in the liver of subordinates, but it decreased in the kidney of subordinates. In contrast, the expression of G6Pase decreased in both tissues of queens during hypoxia (55% in the kidney and 30% in the liver). Finally, the expression of PCK decreased by ~ 40% in the liver of queens but remained unchanged in the kidney during hypoxia.

### 3.4 Figures and tables

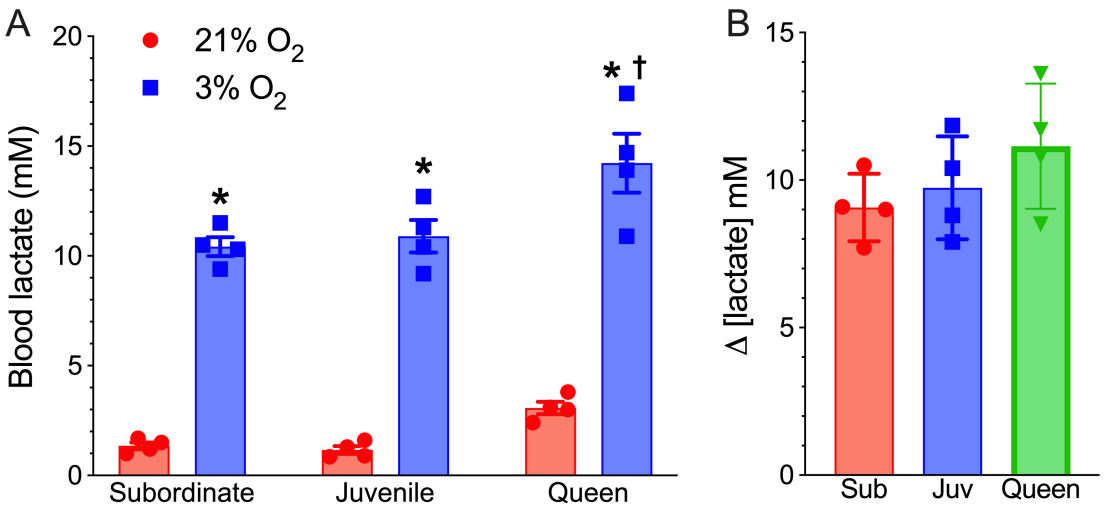


**Figure 3.1 Acute hypoxia increases blood lactate across developmental stages in NMRs** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. (B) Δ change in blood lactate levels between normoxia and hypoxia for each group. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (one and two-way ANOVA, $P < 0.05$).

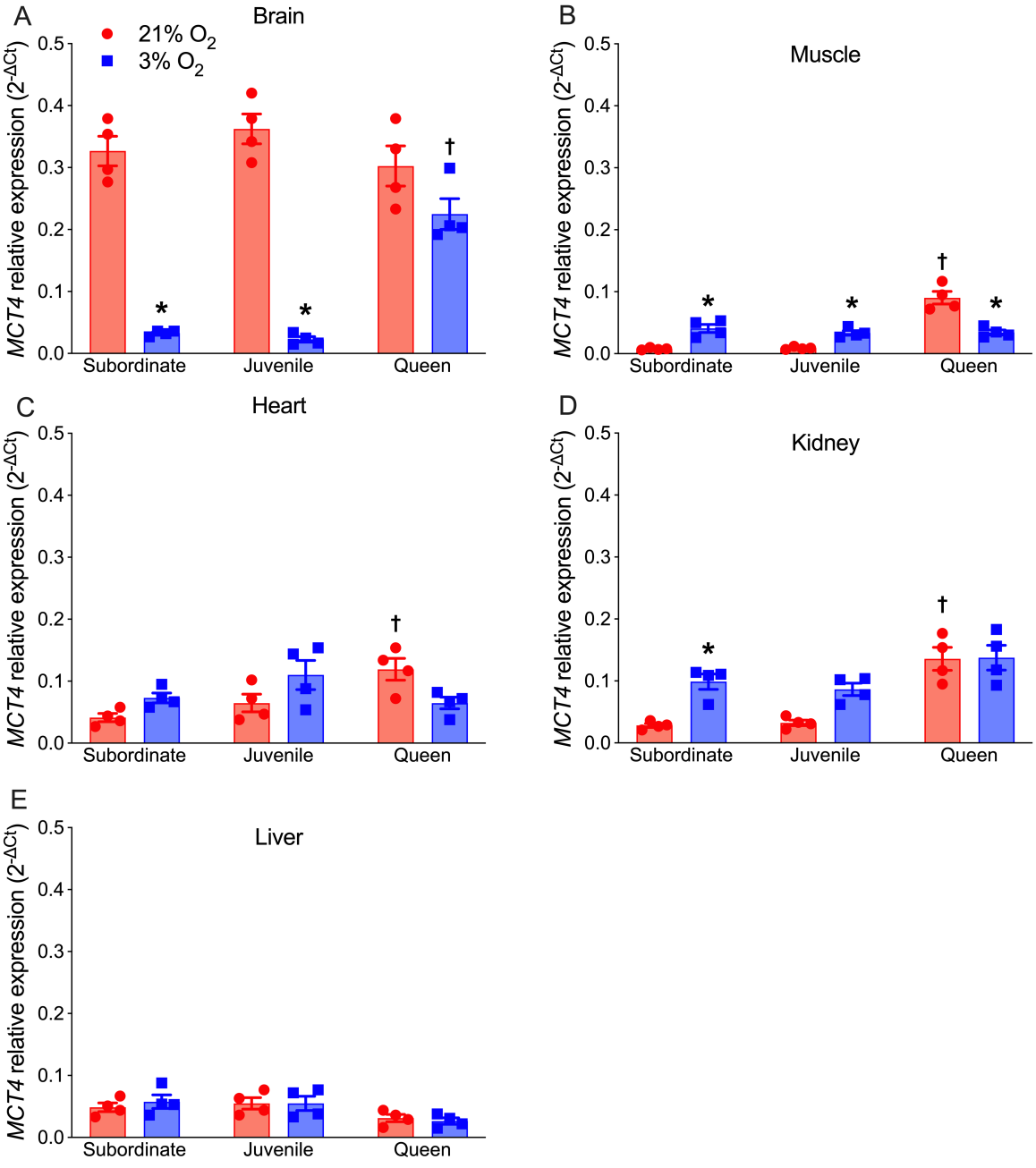


**Figure 3.2 Monocarboxylate transporter 4 (MCT4) gene expression across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. MCT4 gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

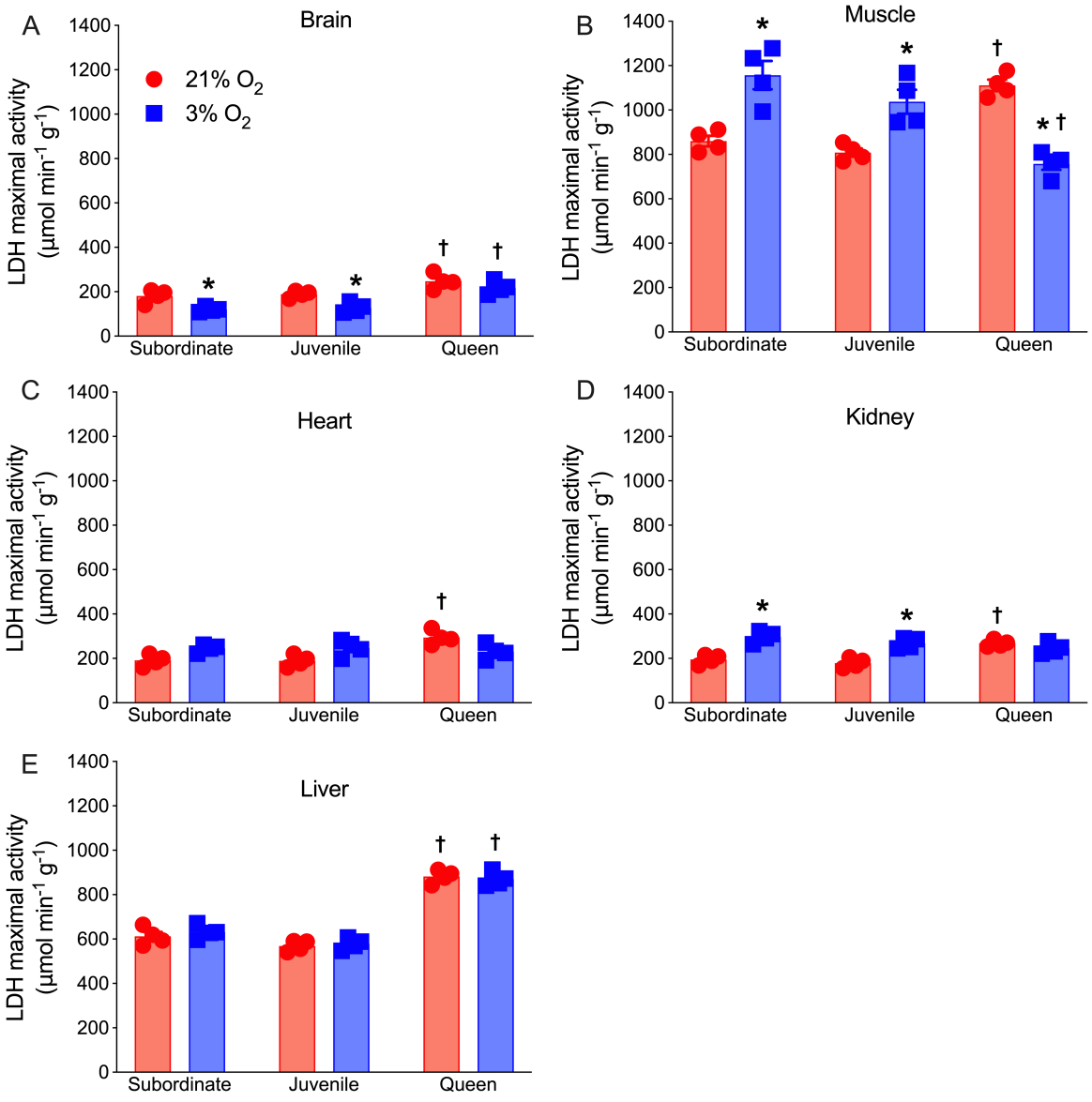


**Figure 3.3 Lactate dehydrogenase (LDH) enzyme activity across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. LDH activity per gram of tissue was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

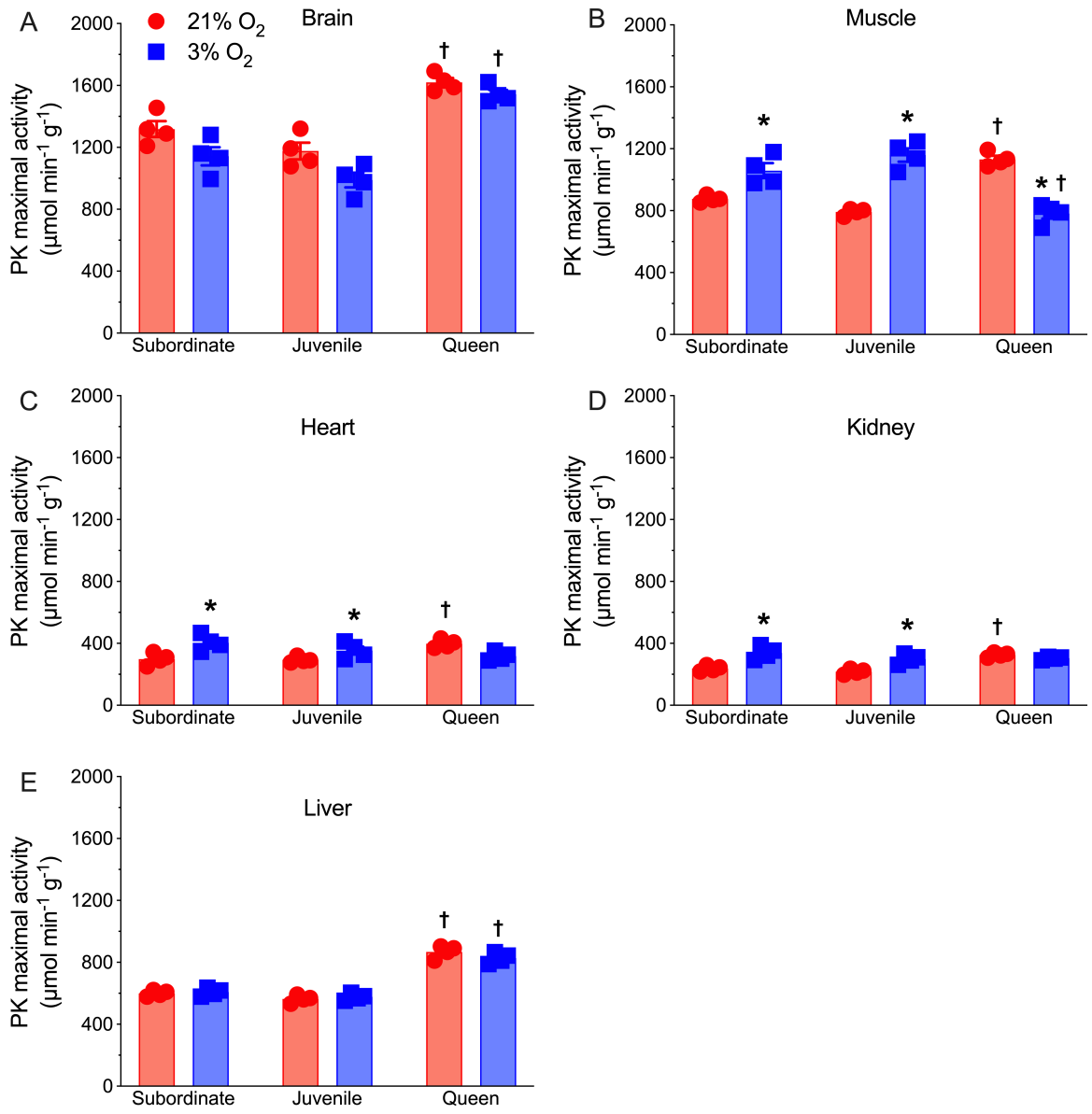


**Figure 3.4 Pyruvate kinase (PK) enzyme activity across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. PK activity per gram of tissue was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

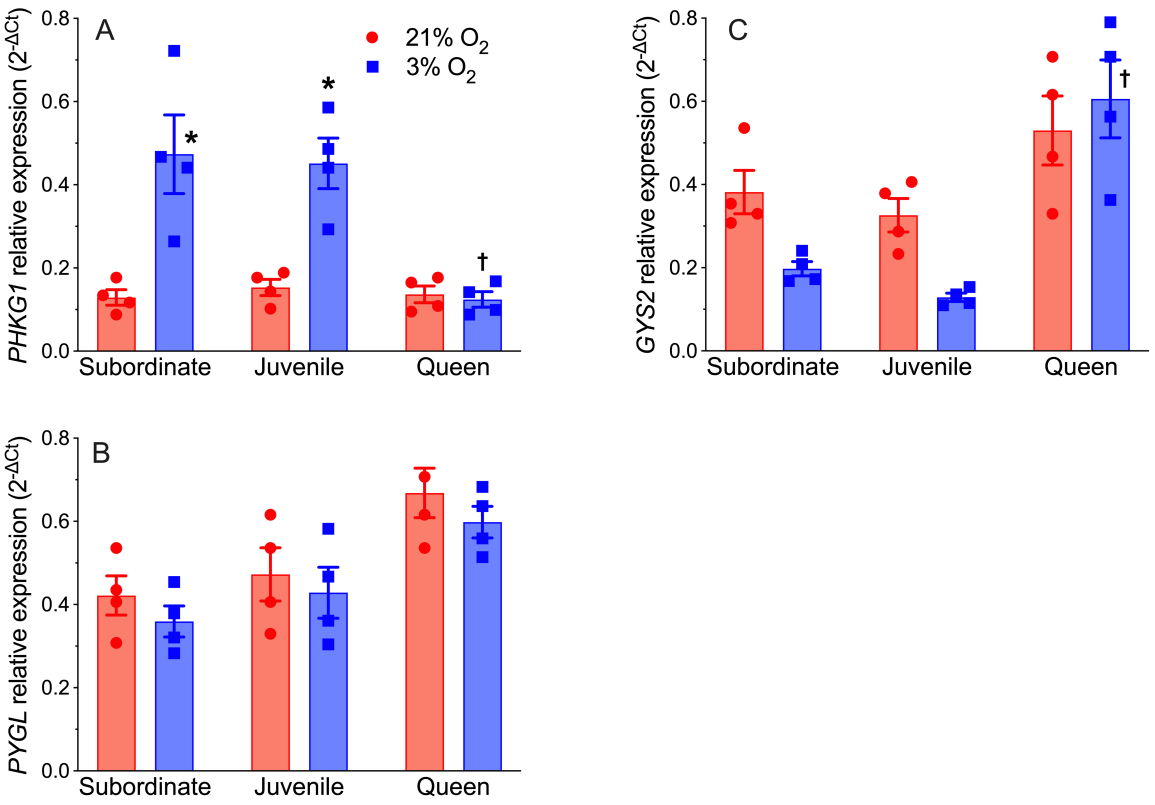


**Figure 3.5 The expression of liver glycogen enzymes is differentially regulated during hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. Expression of the genes for phosphorylase kinase gamma 1 (PHKG1; **A**), liver glycogen synthase 2 (GYS2; **B**) and glycogen phosphorylase (PYGL; **C**), was measured across developmental stages in NMR liver. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

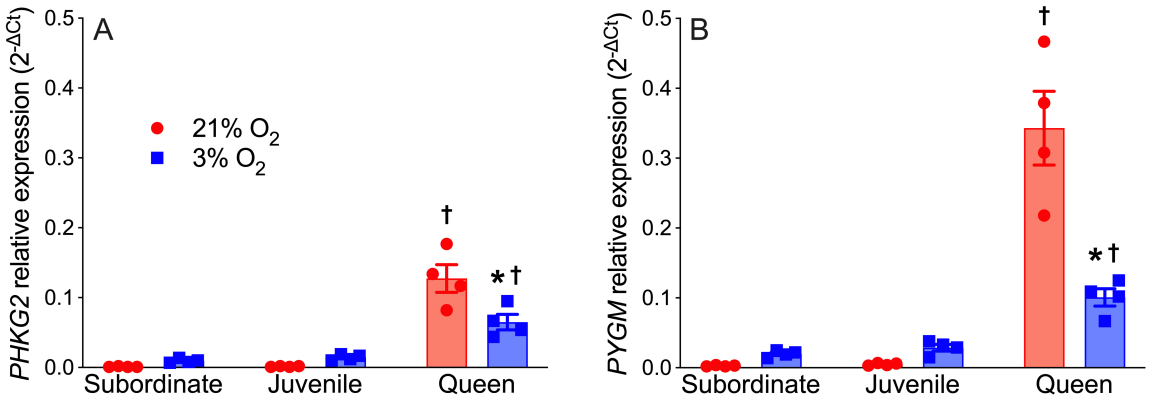

**Figure 3.6 The expression of muscle glycogen enzymes is differentially regulated during hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. The expression of phosphorylase kinase gamma 2 (PHKG2; **A**) and muscle glycogen phosphorylase (PYGM; **B**) genes was measured across developmental stages in NMR skeletal muscle. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

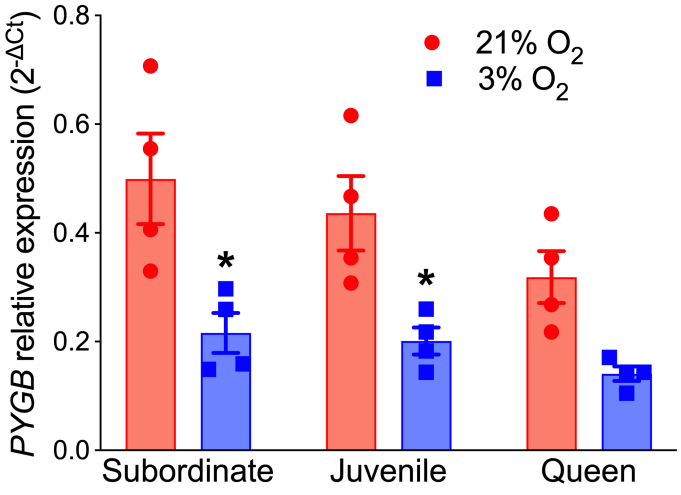


**Figure 3.7 The gene expression of brain glycogen phosphorylase decreases during hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

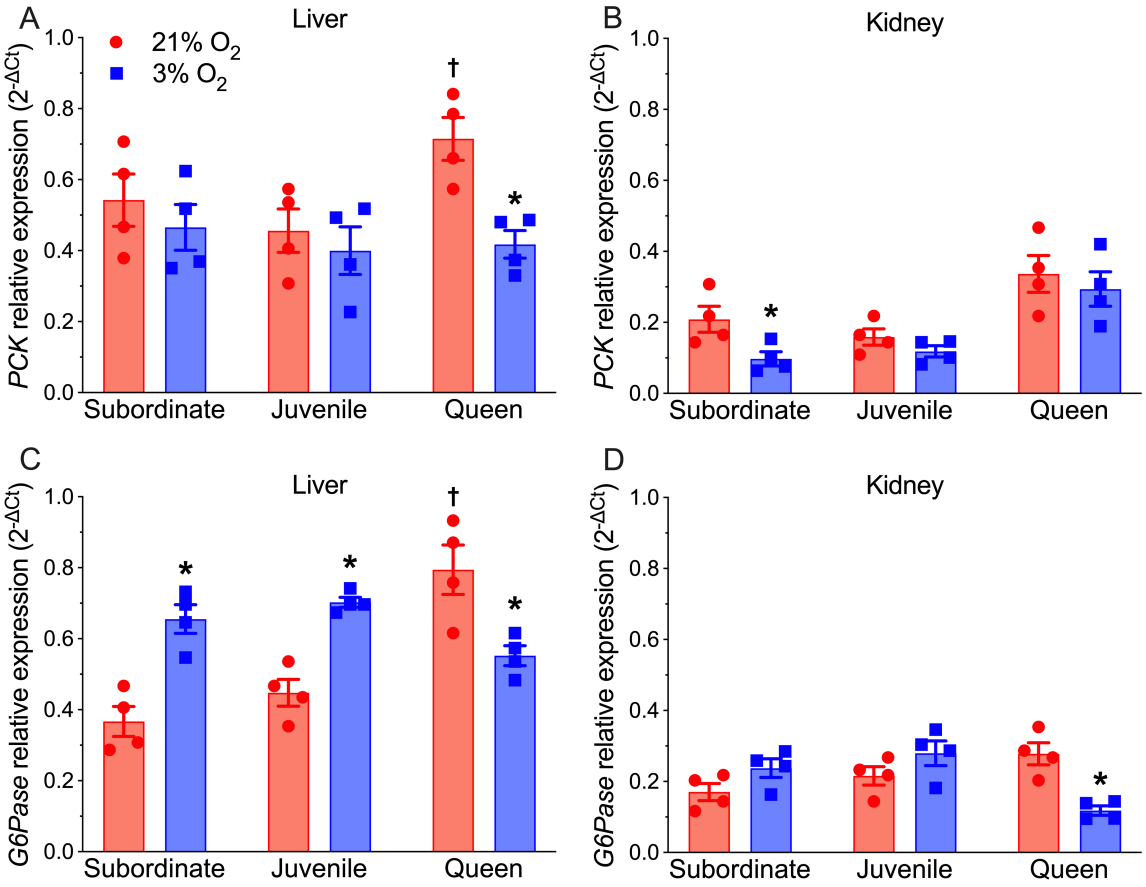


**Figure 3.8 The expression of gluconeogenic enzymes is differentially regulated during hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 h. The expression of phosphoenolpyruvate carboxykinase (PCK) and glucose-6-phosphatase (G6Pase) genes was measured across developmental stages in NMRs: (**A**) G6pase in liver, (**B**) PCK in liver, (**C**) G6pase in kidney, and (**D**) PCK in kidney. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

| Gene | Forward Primer (5' to 3') | Reverse Primer (5' to 3') | Efficiency (%) | $R^2$ |
|---|---|---|---|---|
| MCT4 | CTGTGGATGTGAGGGTGGAC | CTCCCCGTTTTTCTCAGGCT | 96.4 | 0.98 |
| PHKG1 | CTGCTCAGGGGACATGGTTT | GGGGGTGTCTGATCTCTTGC | 98.3 | 0.97 |
| PYGL | GATTGGCTCAGGCATGGAAAC | ACGGGGGTGTCATAAGGGA | 104.5 | 0.94 |
| GYS2 | GGACGCCATGAATAAGCACG | CAGGTTCCAGGCAGAGTAGC | 87.1 | 0.99 |
| PHKG2 | TTTTGGGTTCTCCTGCCACTT | CATAGCCTGGGTGGGTTTCAT | 93.2 | 0.95 |
| PYGM | ACTCATCACTGCCATTGGGG | ATCACCTTTTCAGCCAGCGA | 89.4 | 0.93 |
| PYGB | GAACCTGTGACACTCTCCTGG | AGGCATCTCTGCTTTCTGTCC | 102.6 | 0.97 |
| PCK | CAGCTCACACCCATTGGCTA | TACGTTGACATTCCCCAGGC | 111.4 | 0.95 |
| G6pase | TGTCAGAAGTTGCGTCCTCC | AGCACACCTGGTGAAGTCTC | 96.7 | 0.94 |
| Eukaryotic elongation factor 2 | CTGCCAGCTCATCCTAGACC | CTTGTCCTTGTCCTCGCTGT | 95.3 | 0.97 |
| Actb | CTCTGTGTGGATCGGTGGC | GGGTGAAAGGCAGCGAAGTA | 97.6 | 0.98 |

**Table 3.1 Primer sequences used for qPCR in this study.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 20.68$, $P < 0.0001$ | $F_{2,18} = 8.64$, $P = 0.0023$ | $F_{1,18} = 177.8$, $P < 0.0001$ |
| Heart | $F_{2,18} = 6.94$, $P = 0.0058$ | $F_{2,18} = 3.40$, $P = 0.0558$ | $F_{1,18} = 0.40$, $P = 0.5350$ |
| Liver | $F_{2,18} = 0.32$, $P = 0.7239$ | $F_{2,18} = 5.75$, $P = 0.0117$ | $F_{2,18} = 0.04$, $P = 0.8426$ |
| Muscle | $F_{2,18} = 40.96$, $P < 0.0001$ | $F_{2,18} = 35.48$, $P < 0.0001$ | $F_{1,18} = 0.01$, $P = 0.9114$ |
| Kidney | $F_{2,18} = 3.81$, $P = 0.0417$ | $F_{2,18} = 22.15$, $P < 0.0001$ | $F_{1,18} = 15.69$, $P = 0.0009$ |

**Table 3.2 F-statistics analysis of the effects of hypoxia, caste and their interaction on MCT4 expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on MCT4 expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on MCT4 expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 1.154$, $P = 0.3377$ | $F_{2,18} = 26.94$, $P < 0.0001$ | $F_{1,18} = 24.99$, $P < 0.0001$ |
| Heart | $F_{2,18} = 11.30$, $P = 0.0007$ | $F_{2,18} = 6.138$, $P = 0.0093$ | $F_{1,18} = 1.792$, $P = 0.1973$ |
| Liver | $F_{2,18} = 0.55$, $P = 0.5853$ | $F_{2,18} = 223$, $P < 0.0001$ | $F_{1,18} = 0.18$, $P = 0.6759$ |
| Muscle | $F_{2,18} = 41.05$, $P < 0.0001$ | $F_{2,18} = 2.77$, $P = 0.0891$ | $F_{1,18} = 3.20$, $P = 0.0904$ |
| Kidney | $F_{2,18} = 19.05$, $P < 0.0001$ | $F_{2,18} = 4.43$, $P = 0.0271$ | $F_{1,18} = 37.70$, $P < 0.0001$ |

**Table 3.3 F-statistics analysis of the effects of hypoxia, caste and their interaction on lactate dehydrogenase (LDH) enzyme activity across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on LDH enzyme activity

in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on LDH enzyme activity in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 0.88$, $P = 0.4297$ | $F_{2,18} = 61.82$, $P < 0.0001$ | $F_{1,18} = 15.16$, $P = 0.0011$ |
| Heart | $F_{2,18} = 13.04$, $P = 0.0003$ | $F_{2,18} = 1.83$, $P = 0.1876$ | $F_{1,18} = 3.43$, $P = 0.0841$ |
| Liver | $F_{2,18} = 2.1$, $P = 0.1514$ | $F_{2,18} = 222$, $P < 0.0001$ | $F_{1,18} = 0.31$, $P = 0.5821$ |
| Muscle | $F_{2,18} = 70.97$, $P < 0.0001$ | $F_{2,18} = 0.18$, $P = 0.8302$ | $F_{1,18} = 6.85$, $P = 0.0174$ |
| Kidney | $F_{2,18} = 14.54$, $P = 0.0002$ | $F_{2,18} = 10.74$, $P = 0.0009$ | $F_{1,18} = 28.77$, $P < 0.0001$ |

**Table 3.4 F-statistics analysis of the effects of hypoxia, caste and their interaction on pyruvate kinase (PK) enzyme activity across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on PK enzyme activity in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on PK enzyme activity in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Liver (PHKG1) | $F_{2,18} = 8.01$, $P = 0.0032$ | $F_{2,18} = 8.33$, $P = 0.0027$ | $F_{1,18} = 28.24$, $P < 0.0001$ |
| Liver (PYGL) | $F_{2,18} = 0.03$, $P = 0.9679$ | $F_{2,18} = 11.67$, $P = 0.0006$ | $F_{1,18} = 1.86$, $P = 0.1891$ |
| Liver (GYS2) | $F_{2,18} = 3.49$, $P = 0.0521$ | $F_{2,18} = 19.35$, $P < 0.0001$ | $F_{1,18} = 4.59$, $P = 0.0459$ |
| Muscle (PHKG2) | $F_{2,18} = 10.38$, $P = 0.0010$ | $F_{2,18} = 61.83$, $P < 0.0001$ | $F_{1,18} = 3.24$, $P = 0.0886$ |
| Muscle (PYGM) | $F_{2,18} = 23.22$, $P < 0.0001$ | $F_{2,18} = 58.08$, $P < 0.0001$ | $F_{1,18} = 13.62$, $P = 0.0017$ |
| Brain (PYGB) | $F_{2,18} = 0.51$, $P = 0.6041$ | $F_{2,18} = 3.197$, $P = 0.0649$ | $F_{1,18} = 29.77$, $P < 0.0001$ |

**Table 3.5 F-statistics analysis of the effects of hypoxia, caste and their interaction on glycolytic enzyme expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on glycolytic enzyme expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on glycolytic enzyme expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Liver (PCK) | $F_{2,18} = 2.33$, $P = 0.1254$ | $F_{2,18} = 2.50$, $P = 0.1095$ | $F_{1,18} = 8.064$, $P = 0.0109$ |
| Kidney (PCK) | $F_{2,18} = 0.64$, $P = 0.5389$ | $F_{2,18} = 15.30$, $P = 0.0001$ | $F_{1,18} = 5.02$, $P = 0.0379$ |
| Liver (G6pase) | $F_{2,18} = 24.79$, $P < 0.0001$ | $F_{2,18} = 7.49$, $P = 0.0043$ | $F_{1,18} = 8.46$, $P = 0.0093$ |
| Kidney (G6pase) | $F_{2,18} = 11.76$, $P = 0.0005$ | $F_{2,18} = 2.04$, $P = 0.1586$ | $F_{1,18} = 0.18$, $P = 0.6692$ |

**Table 3.6 F-statistics analysis of the effects of hypoxia, caste and their interaction on gluconeogenic enzyme expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on gluconeogenic enzyme expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on gluconeogenic enzyme expression in the respective tissue**.**

### 3.5 Discussion

Our study provides insight into metabolic adaptations of NMRs to hypoxia and distinguishes divergent underlying regulatory strategies across developmental stages and hierarchical status within the colony. Our results provide several important advances in our understanding of how NMRs adapt to life in hypoxia, and how this is shaped by their social structure in their natural ecological niche. First, we found that blood lactate levels increase during severe hypoxia across all developmental groups. In isolation, this finding suggests that all developmental stages of NMRs upregulate anaerobic metabolism in severe hypoxia (3% $O_2$). However, our exploration of the underlying genes and enzymes involved in anaerobic metabolism reveal a divergent and tissue-specific phenotype across development and caste, which supports our initial hypothesis, such that subordinate juveniles and adults have largely the same response to hypoxia, and queens employ a different strategy.

Specifically, subordinates and juveniles upregulate MCT4 expression and increase the activities of glycolytic enzymes in muscle and kidney, with a trend towards a similar response in heart tissue. Conversely, measurements in liver reveal no change in MCT4 expression or glycolytic enzyme activity, suggesting no increase in anaerobic metabolism in this tissue. However, markers of glycogenolysis are upregulated, consistent with mobilization of liver glycogen stores to support blood glucose supply. We also report evidence of increased glucogenesis in liver with hypoxia. Together, these changes are expected to result in increased lactate export from cells to the blood, increased tissue (and particularly muscle) glycolytic activity, and mobilization of glucose from liver concomitant with elevated gluconeogenesis in the liver and kidney (29, 47, 197-199). Intriguingly, an opposing phenotype was observed in brain, where hypoxia led to decreased mRNA expression of MCT4, and glycogenolysis and LDH enzyme activity. Taken together, and with the

notable exception of brain tissue, these findings support increased reliance on anaerobic metabolism to meet energy demands and an ability to cycle glucose stores to sustain the supply of metabolic fuel and support carbohydrate metabolism in hypoxia.

This phenotype may reflect the roles and responsibilities of non-reproductive NMRs within the colony hierarchy. Specifically, subordinate juvenile and adult NMRs are very active within a burrow/colony setting, and regularly engage in intense aerobic work, including burrowing, tunnel maintenance, fighting off predators, searching for and transporting food, etc. Our findings suggest that juvenile and adult subordinates upregulate anaerobic metabolism in muscle, and to a lesser extent in kidney, to sustain activity-related energy demands associated with these tasks, even in hypoxia. Thus, the systemic increase in blood lactate in these developmental stages is likely due to increased anaerobic metabolism in exercising muscle.

In contrast, queens appear to have a greater reliance on anaerobic metabolism in normoxia but do not markedly upregulate systemic use of anaerobic metabolism in hypoxia. Specifically, and in sharp contrast to other developmental stages and castes examined, queens 1) reduce MCT4 expression and glycolytic enzyme activity in muscle, 2) do not increase the expression of glycogenolysis enzymes in liver, but 3) do decrease the expression of glycogenolysis enzymes in muscle, and 4) downregulate the expression of glucogenesis-related enzymes in liver and kidney. In brain, MCT4 expression is decreased but this change is smaller than the reduction observed in juvenile and subordinate adults. In addition, the expression of glycogenolysis enzyme in the brain is maintained. Together, these findings suggest that queens prioritize the energetic demands of brain in hypoxia but otherwise reduce their systemic reliance on anaerobic metabolism relative to their normoxic baseline. Thus, the systemic increase in blood lactate in queens may be the result of sustaining a relatively high level of brain function, supported in part by anaerobic metabolism,

with the accumulated lactate circulating systemically until it can be oxidized following reoxygenation when environmental $O_2$ levels are higher. This strategy may reflect the relatively sedentary lifestyle of queens within the nest of the NMR burrows, which may allow these animals to reduce physical activity (and thus skeletal muscle metabolism) and redirect energy to support the demands of hypoxia-sensitive brain.

***Temporal regulation of glycolysis in hypoxic NMRs***. Intriguingly, previous studies of subordinate NMR muscle metabolism during longer hypoxia exposures indicate a reduction in glucose utilization, which contrasts with our findings in subordinates and juveniles treated with short-term acute hypoxia. Specifically, Hadj-Moussa et al. reported a downregulation of AMPK and its downstream effectors, including GLUT4, during longer-term severe hypoxia (3% $O_2$ for 4 hr) (167). Similarly, Farhat et al. reported a decrease in glycolytic enzyme activity in muscle during chronic moderate hypoxia (11% $O_2$ for 4-6 weeks) (158). Together, these studies suggest that in certain conditions, NMR glucose metabolism may be reduced to conserve energy. However, our findings highlight that glucose metabolism is likely upregulated in subordinates and juveniles during severe acute hypoxia of 1 hr, indicating that glycolytic pathway is dynamically regulated to meet energy demands and the reliance on limited carbohydrate supplies begins to decline with prolonged hypoxia exposure. These differences emphasize the complexity of the regulation of glucose metabolism in NMRs, where the balance between energy production and conservation is finely tuned based on the severity and duration of hypoxic stress.

***Reduced brain metabolism in hypoxia***. An interesting finding in our study pertains to hypoxic brain, where markers of anaerobic metabolism generally trend toward downregulation across

developmental stages and castes. However, not all markers show statistically significant reductions. This pattern of a general reduction in glycolytic activity in brain aligns with previous findings of metabolic suppression in the NMR brain during hypoxia, in which global $Na^+/K^+$-ATPase activity and mitochondrial respiration are each marked reduced by acute or chronic *in vivo* hypoxia exposure (156, 184, 200). The reduction of glycolytic activity, combined with a decrease in MCT4 expression across all groups, suggests that lactate production and export are minimized in brain, likely as a strategy to prevent lactate accumulation and mitigate the risk of acidosis, which could impair neural function (198, 201). The expression of MCT4 in normoxia is much greater in the brain than in other tissues (>6-fold), and its 90% reduction during hypoxia suggests that the brain might employ alternative pathways such as ketone utilization or amino acid metabolism to meet its energy demands and sustain neural activity under low $O_2$ conditions.

In addition, previous reports examining adaptations in the hypoxic brain of subordinate NMRs suggest a shift towards increased reliance on the pentose phosphate pathway, potentially as a protective mechanism to prioritize NADPH production for maintaining redox balance and defending against oxidative stress (185, 202, 203). To support the pentose phosphate pathway, NMRs may increase glucose-6-phosphate supply through enhanced glucose uptake, glycogenolysis, or possibly alternative pathways such as gluconeogenesis or lactate metabolism (204, 205).

***Maintained glycolytic activity in the hypoxic NMR heart.*** Our results indicate that glycolytic enzyme activity in the heart of subordinate, juvenile, and queen NMRs remains unchanged except PK enzyme activity in subordinates, which increased. This finding contrasts with studies showing that NMRs possess enhanced glycogen stores in their cardiac muscle compared to hypoxia-

intolerant mice, presumably to support a greater reliance on glucose utilization under low $O_2$ conditions (187). Similarly, studies on subordinate NMRs in chronic hypoxia (11% $O_2$ for 4-6 weeks) demonstrate that although PK activity decreases, oxidation pathways are upregulated, allowing them to maintain their energy pathways in the heart to meet ATP demands, unlike other tissues where these pathways are suppressed (158). These findings suggest that while NMRs possess large cardiac glycogen reserves, they may not increase cardiac glycolytic activity under severe and acute hypoxia, although they may be able to sustain anaerobic work for longer periods of time.

***Upregulation of genes involved in the liver and muscle, but not the brain***. Acute hypoxia generally increases the expression of enzymes involved in glycogenolysis in the liver and muscle of juveniles and subordinate adult NMRs. Although results show that the expression of PYGL in the liver remains unchanged, the more pronounced increase in the expression of PHKG1 suggests enhanced activation of glycogen phosphorylase through phosphorylation. These changes in the liver are expected to support elevated blood glucose levels during hypoxia (123), and presumably support anaerobic metabolism in vital organs like the brain and heart. Conversely, as skeletal muscle lacks G6Pase, which is required for glucose release into the bloodstream (206, 207), glycogenolysis in muscle serves as a local energy source rather than contributing to systemic glucose levels.

Interestingly, glycogenolysis enzymes in the muscle and liver of queens respond differently to hypoxia than in other groups. For example, the expression of PYGM in subordinates and juveniles is significantly lower than in queens under both normoxic and hypoxic conditions. These differences between groups, combined with the reduction in glycogenolysis enzymes in the muscle

of queens, suggest that queens may prioritize energy conservation in muscle tissue while simultaneously leveraging carbohydrate metabolism. Furthermore, the expression of PHKG1 and PYGL are unchanged in the liver of queens. These data indicate that the liver of queens continues to release glucose into the bloodstream during hypoxia like in normoxic conditions, presumably to support other tissues. Additionally, the lack of change in the expression of the GYS2 enzyme suggests that queens may be replenishing glycogen stores even under hypoxic conditions to maintain metabolic homeostasis.

***Severe acute hypoxia upregulates liver gluconeogenetic genes in subordinates and juveniles, but not queens***. Previous studies demonstrate that gluconeogenic enzymes are expressed at significantly higher levels in the liver than the kidney, underscoring the predominant role of liver in glucose production (208, 209). This is consistent with our findings, which support a central contribution of liver in gluconeogenesis. Specifically, acute hypoxia increases G6Pase expression in the liver of juvenile and subordinate adults, but its expression is reduced in hypoxic queens. G6Pase, the final enzyme in the gluconeogenesis pathway, is responsible for releasing glucose from glucose-6-phosphate (210). In contrast, the expression of PCK, the initial enzyme in gluconeogenesis, is unchanged by hypoxia in the liver and kidney of juvenile and the liver of subordinates, but it is reduced in the kidney of subordinates. The increased G6Pase activity in juveniles and subordinates may reflect a reliance on stored glycogen or non-glucose precursors like glycerol that bypass the need for PCK (211, 212). If this is the case, it would imbue metabolic flexibility that may help NMRs to optimize energy use during hypoxia by avoiding energetically costly pathways like full gluconeogenesis while still maintaining some degree of glucose production to meet systemic demands. Notably, although the observed increase in G6Pase activity

in the liver during hypoxia suggests enhanced glucose mobilization, likely to support systemic energy demands, our results do not directly confirm lactate utilization by the liver. While lactate could potentially serve as a substrate for gluconeogenesis, this remains speculative in the absence of direct evidence. Future studies employing techniques such as isotopic tracing of lactate are necessary to determine whether lactate is actively taken up and utilized by the liver under hypoxic conditions. Nonetheless, it is clear that there is an increased reliance on anaerobic pathways in hypoxia to rapidly generate ATP from glucose in juvenile and adult subordinate NMRs. This may be linked to their ecological role within the colony, where they must sustain very energy-demanding behaviours in variable levels of hypoxia.

It is interesting to consider that we previously reported that subordinate NMRs do not experience metabolic acidosis in hypoxia (123), which is consistent with our current data, as subordinate NMRs increase gluconeogenesis, likely helping to reduce blood lactate levels. This strategy in subordinates and juveniles may help to ensure a continuous supply of glucose to meet energy demands in vital organs while conserving carbon molecules that would otherwise be lost through ventilation (213). This would in turn reduce $CO_2$ production by subordinates and juveniles during hypoxia, which may benefit queens by lowering the overall respiratory burden in crowded and poorly ventilated colony nest chambers and thus helping to stabilize blood pH levels for resident animals. This could also reduce the demand for pH buffering systems, thereby conserving energy (29, 214), and allowing queens to focus resources on reproduction and other vital functions during hypoxia.

***Study limitations***. While our study provides valuable insights into the metabolic responses of NMRs to hypoxia, several limitations should be acknowledged that may limit the conclusions that

may be drawn from our results. First, while qPCR allows for the quantification of transcript levels, these do not always correlate directly with functional protein levels or enzymatic activity, particularly under non-steady-state conditions like acute hypoxia. For instance, post-translational modifications, such as phosphorylation, which can modulate enzyme function independently of transcript expression, were not assessed in this study. Further study is warranted to explore potential roles for post-translational modification in these systems.

Additionally, although we measured plasma lactate levels, the lack of tissue-specific metabolite data, such as tissue-specific measurements of lactate, glycogen, and glucose, limits our ability to draw conclusions about the metabolic contributions of individual tissues during hypoxia. Further experiments in this area would provide greater insight into the role of glycogenolysis, gluconeogenesis, and lactate transport in specific organs. Furthermore, correlating the activities of key glycolytic enzymes, such as LDH and PK, with their respective transcript levels would strengthen the mechanistic interpretations of the observed changes and allow for a deeper exploration of metabolic adaptations to hypoxia across developmental stages and castes in NMRs.

***Conclusions***. Our study provides a comprehensive analysis of tissue-specific metabolic responses to severe acute hypoxia across different developmental stages and castes in NMRs. Our findings indicate that acute hypoxia increases blood lactate levels in subordinate juveniles and adults as a result of enhanced glycolytic pathway activity, primarily in muscle. This likely supports the high glucose demands required for maintaining energy-intensive activities under hypoxic conditions. In contrast, while queens exhibit significantly higher blood lactate levels than other groups under normoxia, they appear to preferentially downregulate metabolic pathways outside of the brain during hypoxia. This suggests that queens prioritize energy conservation in hypoxia more so than

subordinates, potentially to provide greater metabolic capacity to support nursing and reproduction. Unfortunately, we were unable to directly explore this question through analysis of tissues from pregnant or lactating queens because of the devastating impact their removal would have on a given colony.

Nonetheless, our results underscore divergent metabolic adaptations of NMRs in response to hypoxia, highlighting the importance of social rank and developmental stage in shaping strategies of hypoxia tolerance. The observed differences in metabolic responses between queens and non-reproductive animals may reflect the unique ecological and physiological pressures faced by each group. Importantly, these putative links between metabolism and behaviour are speculative; however, the distinct metabolic responses that we observe between castes align with the unique functional demands placed on each caste level and potentially demonstrate how physiological adjustments support colony survival under hypoxic conditions. Additional studies are warranted to explore whether the gene expression an enzyme activity differences described in the present study underlie functional differences in tissue and whole-animal metabolic responses to acute hypoxia across castes.

# Chapter 4: Hypoxia-induced modulation of IGF signaling and glucose transporters in naked mole-rats

This chapter includes material from the following article, which is in preparation for submission to the Journal of Comparative Biochemistry and Physiology Part A:

Ojaghi, M., & Pamenter, M.E. (2025). Hypoxia-induced modulation of IGF signaling and glucose transporters in naked mole-rats

## 4.1 Introduction

In previous chapters, we demonstrated that NMRs increase their blood glucose and lactate levels and regulate energy pathways differently across various tissues, developmental stages, and caste status to survive hypoxia. In this chapter, we focus on how they modulate the stimulators of blood glucose uptake under hypoxic conditions.

Previous studies showed that it is unclear how insulin signaling contributes to hypoxia adaptation in NMRs. Mutations in the insulin β-chain of NMRs compared with other mammals (140) may lead to a form of insulin resistance. However, studies suggest that IGF signaling pathways might play a significant role in modulating glucose metabolism in NMRs (140, 179). IGF-1 typically regulates glucose metabolism in mammals by promoting glucose uptake and enhancing insulin sensitivity (215, 216). Unlike in most mammals, where IGF-2 is primarily expressed during fetal development (217), IGF-2 continues to be expressed in adult NMR tissues (140). In mammals, insulin is not produced by the pancreas until after birth, so during fetal development, glucose regulation is primarily managed by IGF-2, which closely resembles insulin and can bind to insulin receptors (177, 178). In NMRs, this fetal-like mode of glucose regulation may persist into adulthood, where IGF-2 might compensate for the reduced effectiveness of insulin arising due to mutations in the insulin β-chain. The role of IGF signaling in the suppression of metabolic rate in NMRs during hypoxia has not been thoroughly studied, and how this pathway is regulated across different tissues remains largely unexplored.

As we discussed in the first chapter, glucose and fructose utilization in NMRs varies under different $O_2$ levels and exposure durations. In normoxia, GLUT5 expression is significantly higher across tissues in NMRs compared to mice, indicating that NMRs are better adapted to utilize fructose as a metabolic fuel (122). Fructose supports heart and brain function in NMRs during

anoxia, whereas mice are unable to sustain function under such conditions (122). However, during hypoxia (7% $O_2$ for 4 hrs), studies show that GLUT5 and KHK (a key enzyme for fructose metabolism) are downregulated in the brain of NMRs (127). Furthermore, studies on skeletal muscle of NMRs in severe hypoxia (3% $O_2$ for 4 hr) indicate that GLUT4 and p-GS are downregulated, reducing glucose uptake and glycogen storage. Meanwhile, GLUT1 and acetyl-CoA carboxylase (ACC1) remain unchanged, indicating that lipid metabolism in skeletal muscle is not significantly impacted (167). Overall, these studies demonstrate that fuel utilization varies across different tissues in NMRs depending on $O_2$ levels and exposure durations, suggesting that their physiology and cellular mechanisms have evolved to balance energy metabolism through both aerobic and anaerobic pathways.

To explore this, we investigated the extent to which IGF signaling pathways contribute to glucose regulation during severe hypoxia across key tissues and developmental stages in NMRs. We measured the expression of IGF-1, IGF-2, their receptors, the insulin receptor, and glucose and fructose transporters in animals exposed to either normoxia or severe hypoxia (3% $O_2$).

We hypothesized that severe acute hypoxia (3% $O_2$) reduces IGF expression to limit glucose uptake in most tissues, a response that could contribute to metabolic rate suppression. We predicted that: 1) IGF-1 and IGF-2 expression would decrease in tissues; 2) insulin, IGF receptors, and glucose or fructose transporters involved in cellular uptake would be downregulated in some tissues to conserve energy. Based on differences in developmental stage and caste-specific behaviors, we predicted that juvenile and subordinate NMRs would exhibit less downregulation of IGF signaling and glucose uptake compared to queens. This reflects their functional roles in the colony: juveniles and subordinates are primarily engaged in energetically demanding tasks like foraging and tunneling, while queens exhibit reduced activity levels, spending most of their time

in the nest to support reproductive functions. To assess these predictions, we used the samples from the same animals that were used in chapter 3.

## 4.2 Materials and Methodology

### 4.2.1 Animals and experimental design

We used the same sample collected in chapter 2 for our experiments. Briefly, the sample set consisted of 24 NMRs, including 8 adult subordinates (1–13 years; 55 ± 10 g), 8 queens that had previously bred successfully but were neither pregnant nor lactating at the time of the experiment (3–12 years; 65 ± 8 g), and 8 juveniles (~2 months old; 13 ± 2 g). The animals were randomly and evenly assigned to either a normoxic control group or a hypoxic (3% $O_2$) treatment group.

Total RNA was isolated from 50–100 mg tissues, including brain, heart, liver, muscle and kidney, using TRIzol LS reagent, and cDNA was synthesized with the iScript™ cDNA Synthesis Kit, then stored at −20°C. qPCR was performed using Maxima SYBR Green qPCR Master Mix in 10 µl reactions. Primer sequences are in Table 4.1. Samples were run in duplicate with no-template and no-RT controls.

Statistical analyses were performed as described in chapter 3, using GraphPad Prism. A two-way ANOVA assessed intraspecific differences between normoxic and hypoxic (3% $O_2$) conditions, with Šidák's test for post-hoc comparisons ($P<0.05$). Data are presented as mean ± s.e.m., with significant differences indicated by asterisks in figures.

### 4.2.2 Measurement of protein expression by Western blot

Frozen tissues (50-100 mg) were powdered using a mortar and pestle. Samples were then homogenized using sonication (20 pulses, 1 second per pulse) at a concentration of 100 mg/ml (W/V) in RIPA buffer containing both protease and phosphatase inhibitors. The homogenate was centrifuged at 12,000g for 10 mins at 4°C. The supernatant was then collected for protein concentration measurements using the Biuret assay. For SDS-PAGE, 10 µl was loaded into each

lane of a 10% or 12% SDS-polyacrylamide gel, which was run at 130 V for 1–1.5 hrs. Proteins were subsequently transferred to a nitrocellulose membrane at 100 V for 1 hr. The success of the transfer was confirmed with Ponceau S staining. Membranes were blocked for 60 mins with 5% bovine serum albumin to prevent non-specific binding. The membranes were rinsed three times for 5 mins each in TBS-T, followed by overnight incubation at 4°C with the primary antibody. The following antibodies were used: GLUT1 (ABclonal, catalog no. A6982); GLUT4 (ABclonal, catalog no. A7637); GLUT5 (ABclonal, catalog no. A13650). After washing again, membranes were incubated with an HRP-conjugated secondary antibody (Rabbit IgG, Thermofisher, catalog no. AS014) for 1 hr, followed by a final set of three washes. Protein bands were visualized using Clarity™ ECL substrate (Bio-Rad) and imaged with the Bio-Rad ChemiDoc™ XRS+ system. Target protein intensities were measured and normalized to total protein using ImageJ Software (V1.53j, NIH, Bethesda, MD). Normalized values were averaged for the normoxia group, and all values (normoxia and hypoxia) were standardized by dividing them by this average to determine relative expression under hypoxia.

## 4.3 Results

***Severe acute hypoxia alters IGF expression differently across developmental stages***. Analysis with a two-way ANOVA revealed a significant difference in IGF expression between normoxia and hypoxia across all developmental stages. In subordinates, IGF-1 expression exhibited tissue-specific responses: a twofold increase in the heart, a 50% reduction in the brain, and no significant changes in other tissues. Juveniles and queens showed no hypoxia-induced changes in IGF-1 expression across all examined tissues (Fig 4.1 & Table 4.2, n = 4 for each group and treatment). Additionally, there is a significant difference between queens and subordinates in the normoxic brain, hypoxic heart and hypoxic kidney.

IGF-2 expression decreased by over 75% in the brains of subordinates and juveniles, while remaining stable in other tissues except for a significant increase in subordinate muscle (Fig 4.2 & Table 4.3, n = 4 for each group and treatment). Queens displayed an 84% reduction in muscle IGF-2 expression, with no significant changes in other tissues despite observed decreases (~ 70%) in the heart and kidney ($P > 0.05$). Comparison of developmental stage groups indicates a significant difference between queens and subordinates in the hypoxic heart, liver, muscle, kidney and normoxic muscle.

***Divergent changes in the expression of IGF and insulin receptors across developmental stages during severe acute hypoxia***. Subordinates and juveniles exhibited a 90% reduction in insulin receptor expression in the brain and heart, with no changes in other tissues (Fig 4.3 & Table 4.4, n = 4 for each group and treatment). In queens, insulin receptor expression decreased in the heart (85%), muscle (50%) and kidney (50%), while remaining unchanged in the brain and liver.

Comparison across developmental stage groups reveals significant differences between queens and subordinates in all normoxic tissues, except the brain.

IGF-1 receptor expression decreased by 85% in the brain of subordinates and juveniles, while remaining unchanged in other tissues (Fig 4.4 & Table 4.5, n = 4 for each group and treatment). In contrast, queens showed no hypoxia-induced changes in IGF-1 receptor expression across all tissues. However, there are significant differences between queens and subordinates in the normoxic heart, muscle and kidney.

IGF-2 receptor expression remained unchanged across all groups except in queen hearts, where it decreased significantly. Subordinates and juveniles showed a ~ 75% reduction in brain IGF-2 receptor expression, though this did not reach statistical significance (Fig 4.5 & Table 4.6, n = 4 for each group and treatment). However, there are significant differences between queens and subordinates in the normoxic heart, muscle and kidney, as well as in the hypoxic brain and muscle.

***Tissue-specific glucose and fructose transporter expression in NMRs under severe acute hypoxia***. Subordinates exhibited increased GLUT1 mRNA expression in the heart (~ 3-fold) and kidney (~ 4.6-fold), with no significant changes in other tissues. In juveniles and queens, it remained unchanged in all tissues except the muscle of queens where it decreased by 60%. While notable changes in GLUT1 expression occurred in some tissues (e.g., juveniles: a two-fold increase in the heart and 3.5-fold increase in the kidney; queen: a 90% decrease in the heart), these differences did not reach statistical significance (Fig 4.6 & Table 4.7, n = 4 for each group and treatment). Comparison across developmental stage groups reveals significant differences between

queens and subordinates in the hypoxic heart and normoxic muscle. By contrast, GLUT1 protein levels remained unchanged in all groups (Fig 4.9 & Table 4.8, n = 4 for each group and treatment).

GLUT4 mRNA expression showed no significant changes across tissues in subordinates and juveniles. While a 2.5-fold increase was observed in the heart and notable elevations occurred in muscle (4.4-fold in subordinates; 2.5-fold in juveniles), these changes did not reach statistical significance (Fig 4.7 & Table 4.9, n = 4 for each group and treatment). However, GLUT4 protein levels increased only in the heart by 30% in subordinates and juveniles with no change in other tissues (Fig 4.7 & Table 4.10, n = 4 for each group and treatment).

In queens, GLUT4 mRNA expression decreased in the heart, muscle and kidney but there was no change in the brain and liver (Fig 4.7 & Table 4.9). The highest reduction was in the heart (83%), and muscle (60%). However, GLUT4 protein levels decreased only in the muscle and remained unchanged in other tissues (Fig 4.10 & Table 4.10). Comparison of mRNA expression across developmental stage groups shows that significant differences between queens and subordinates in the normoxic heart, muscle and kidney, as well as in the hypoxic muscle

The expression of GLUT5 mRNA decreased in the brain of all groups and in the muscle of queens but remained unchanged in the other groups (Fig 4.8 & Table 4.11, n = 4 for each group and treatment). The protein levels of GLUT5 in subordinates and juveniles remained unchanged in all tissues, except the muscle of juveniles, where they decreased by 38%. In queens, GLUT5 protein levels decreased by ~ 25% in the brain, muscle and liver, but remained unchanged in the heart and kidney (Fig 4.11 & Table 4.12, n = 4 for each group and treatment). Comparison of mRNA expression across developmental stage groups shows that significant differences between queens and subordinates in the normoxic muscle and kidney.

## 4.4 Figures and tables

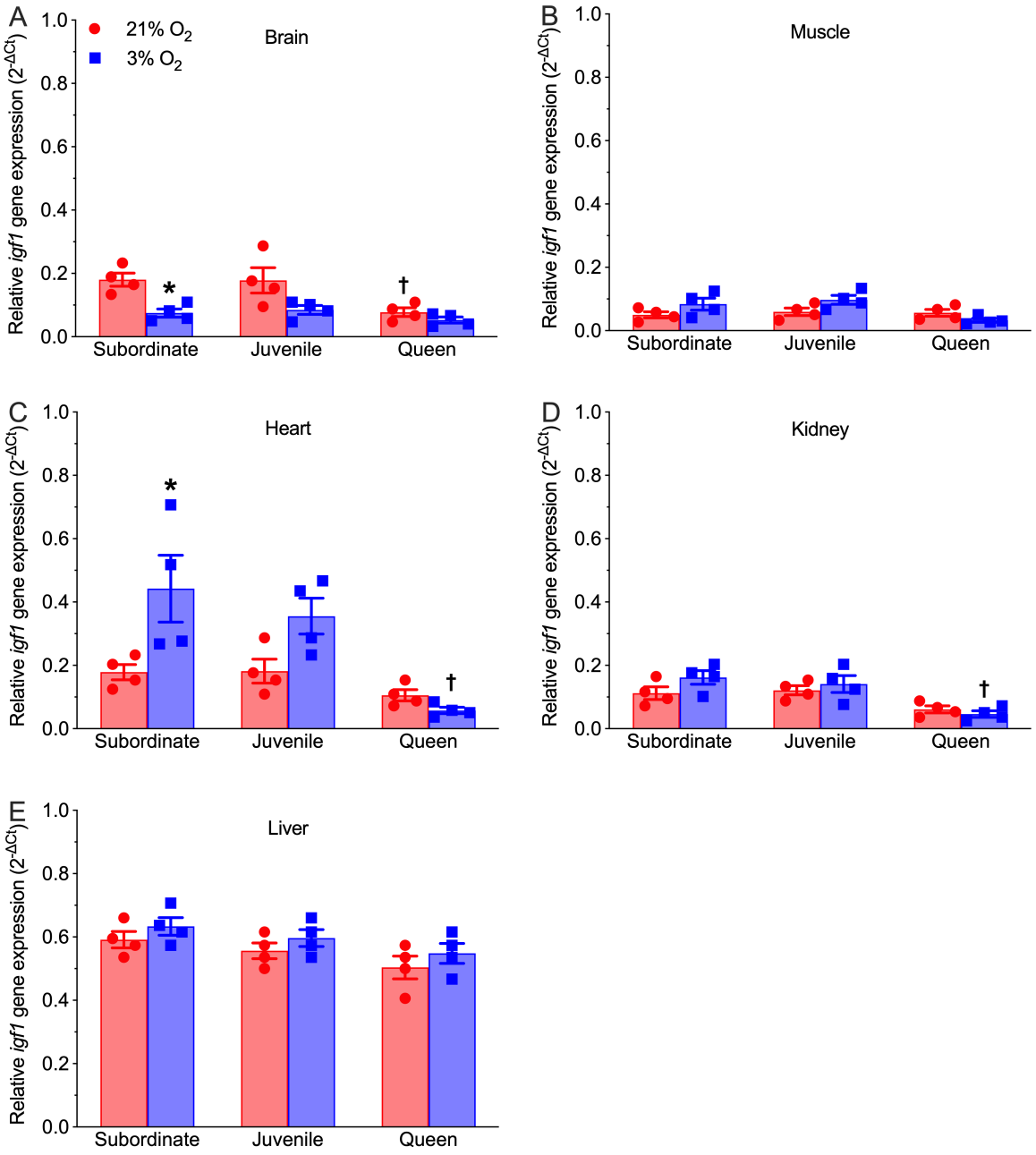


**Figure 4.1 The gene expression of insulin-like growth factor 1 (IGF-1) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. IGF-1 gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each

caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

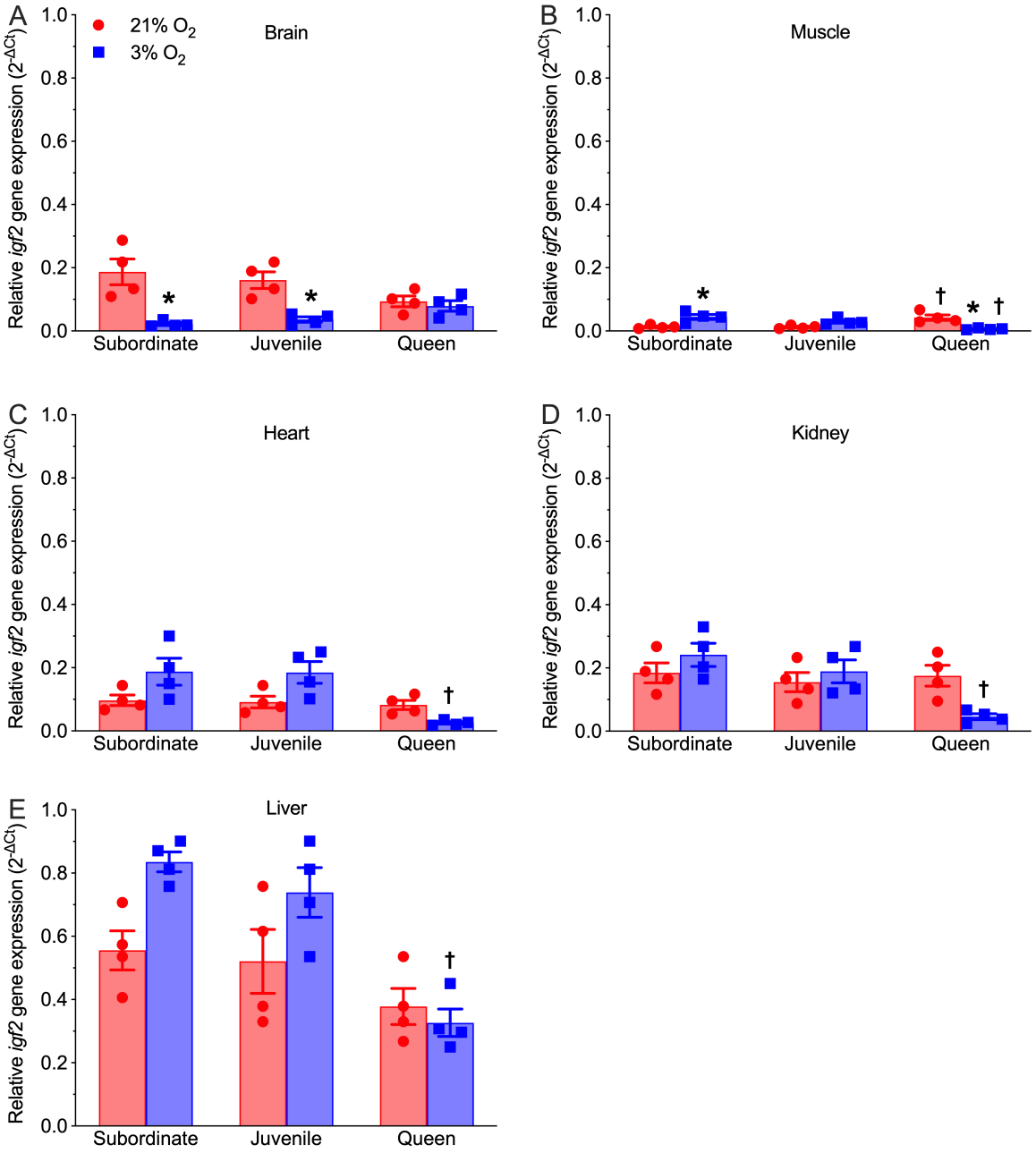


**Figure 4.2 The gene expression of insulin-like growth factor 2 (IGF-2) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. IGF-2 gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

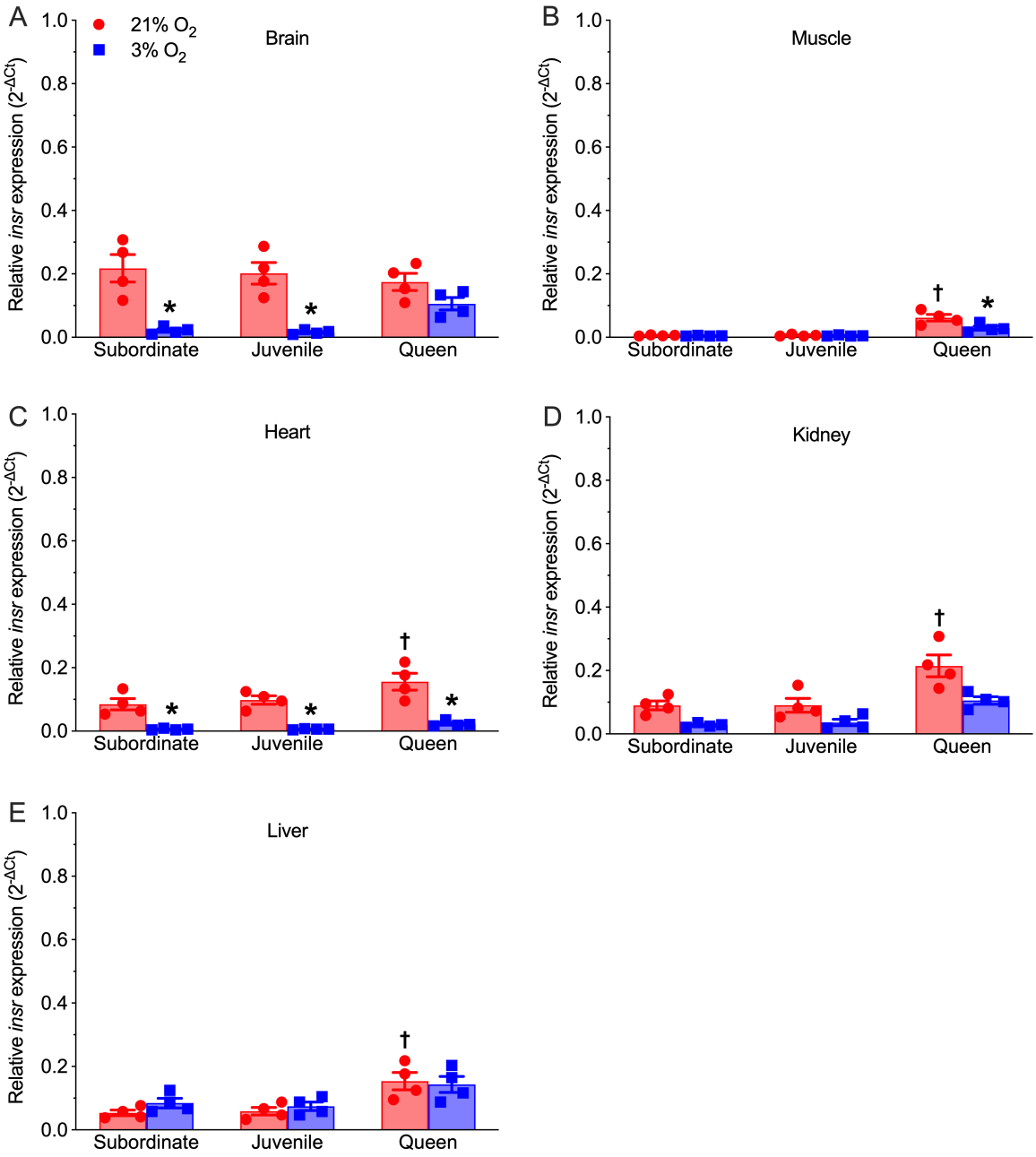


**Figure 4.3 The gene expression of insulin receptor (INSR) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. INSR gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

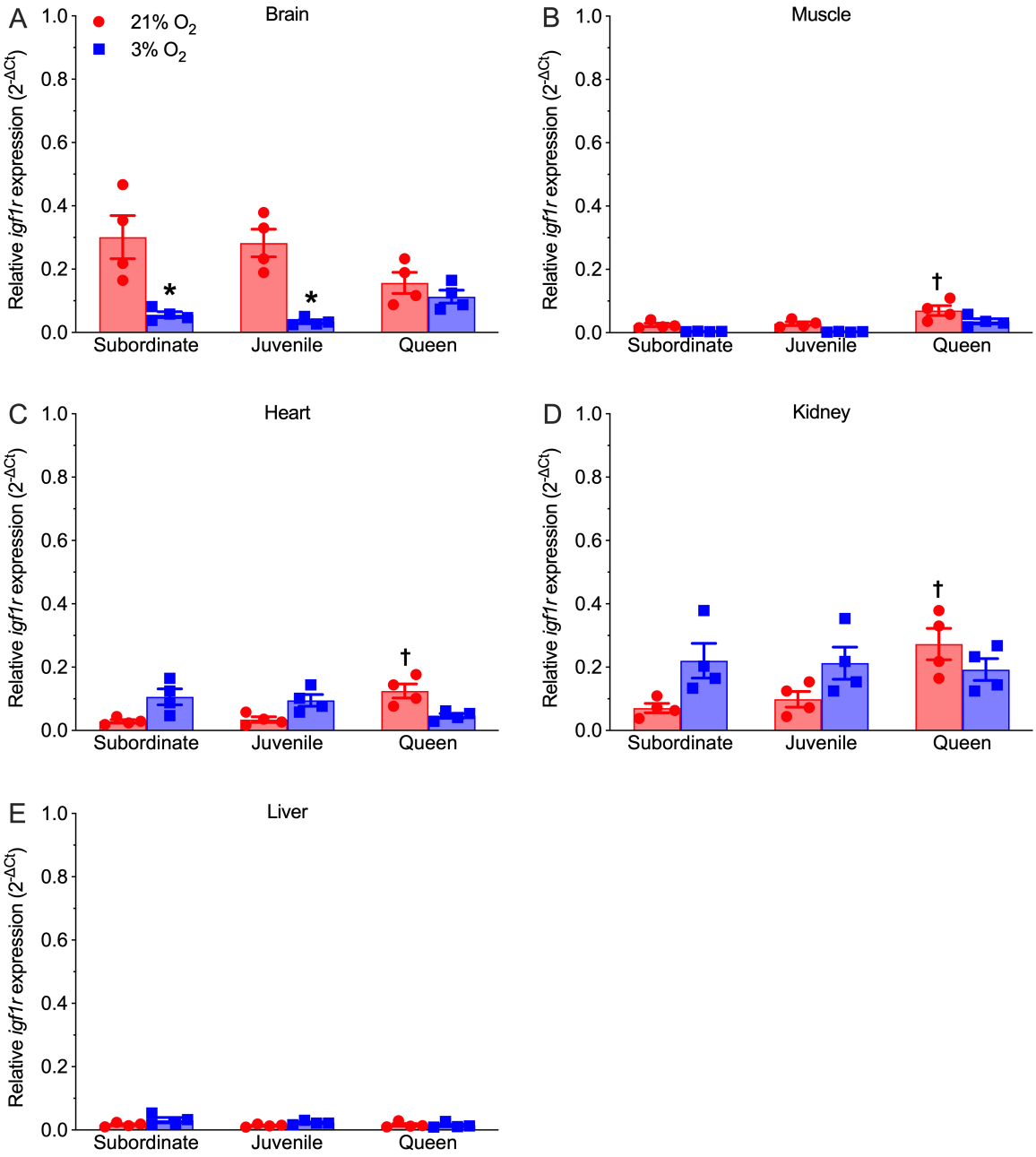


**Figure 4.4 The gene expression of insulin-like growth factor 1 receptor (IGF1R) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. IGF1R gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

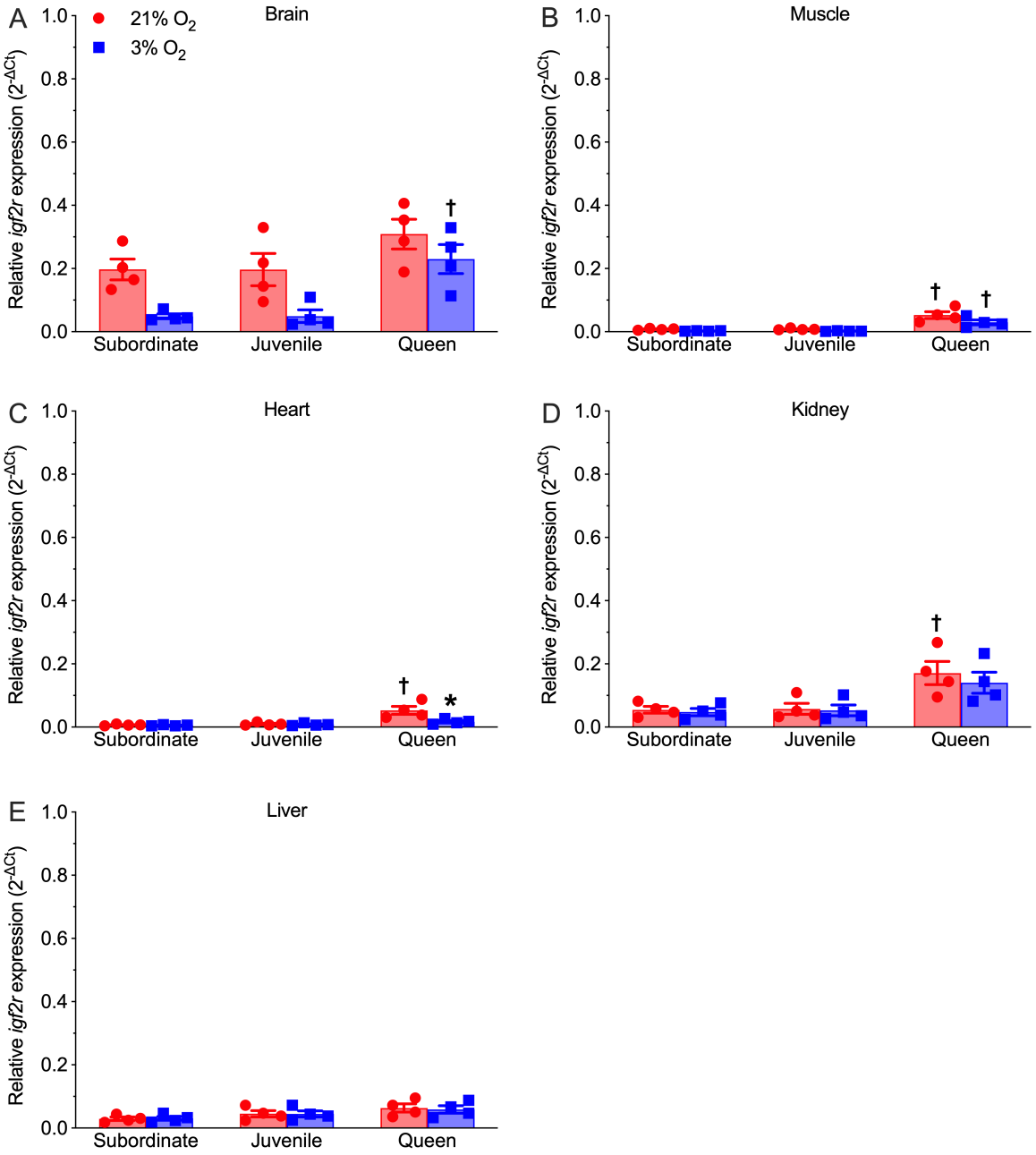


**Figure 4.5 The gene expression of insulin-like growth factor 2 receptor (IGF2R) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. IGF2R gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

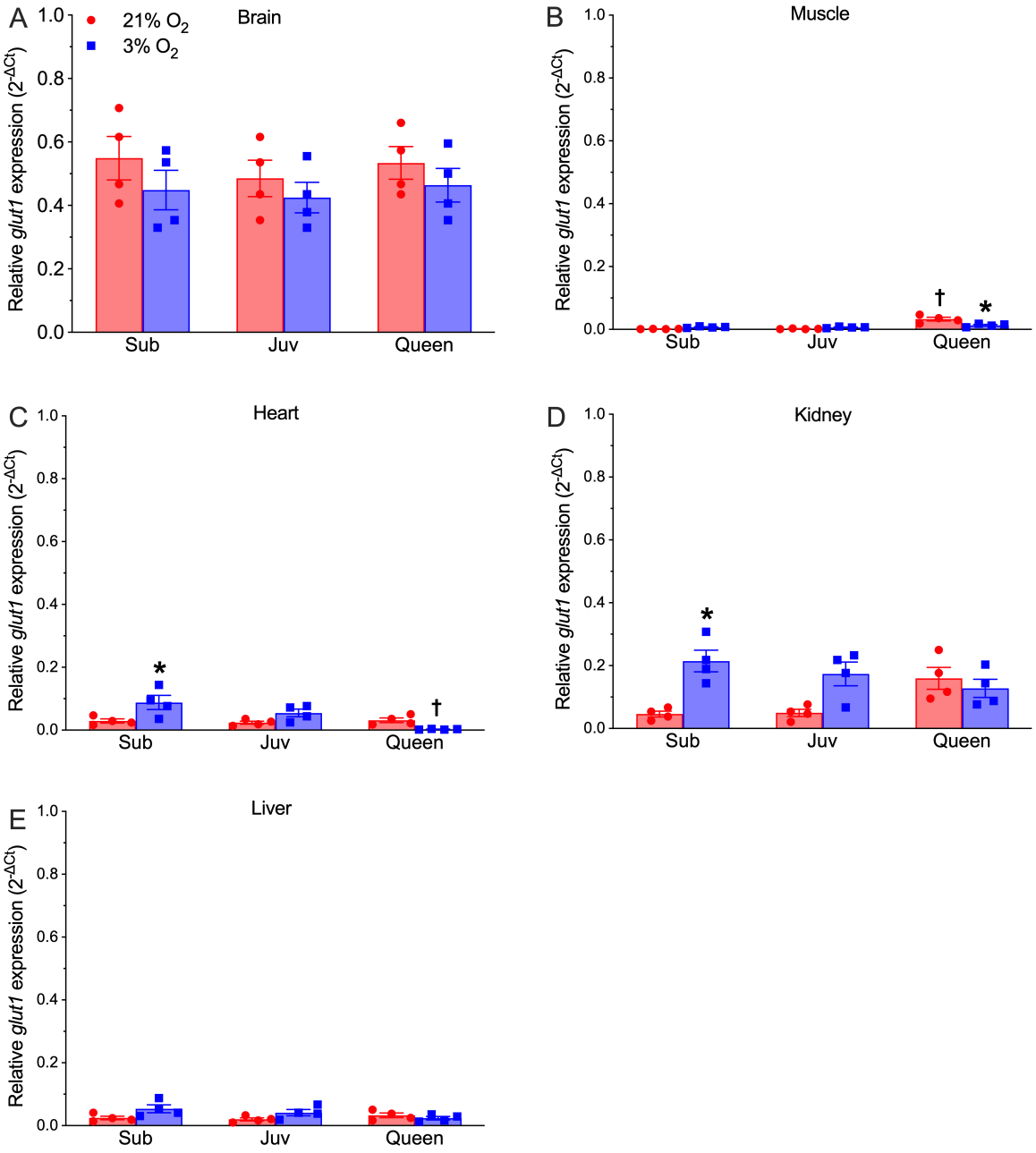


**Figure 4.6 The gene expression of glucose transporter 1 (GLUT1) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. GLUT1 gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

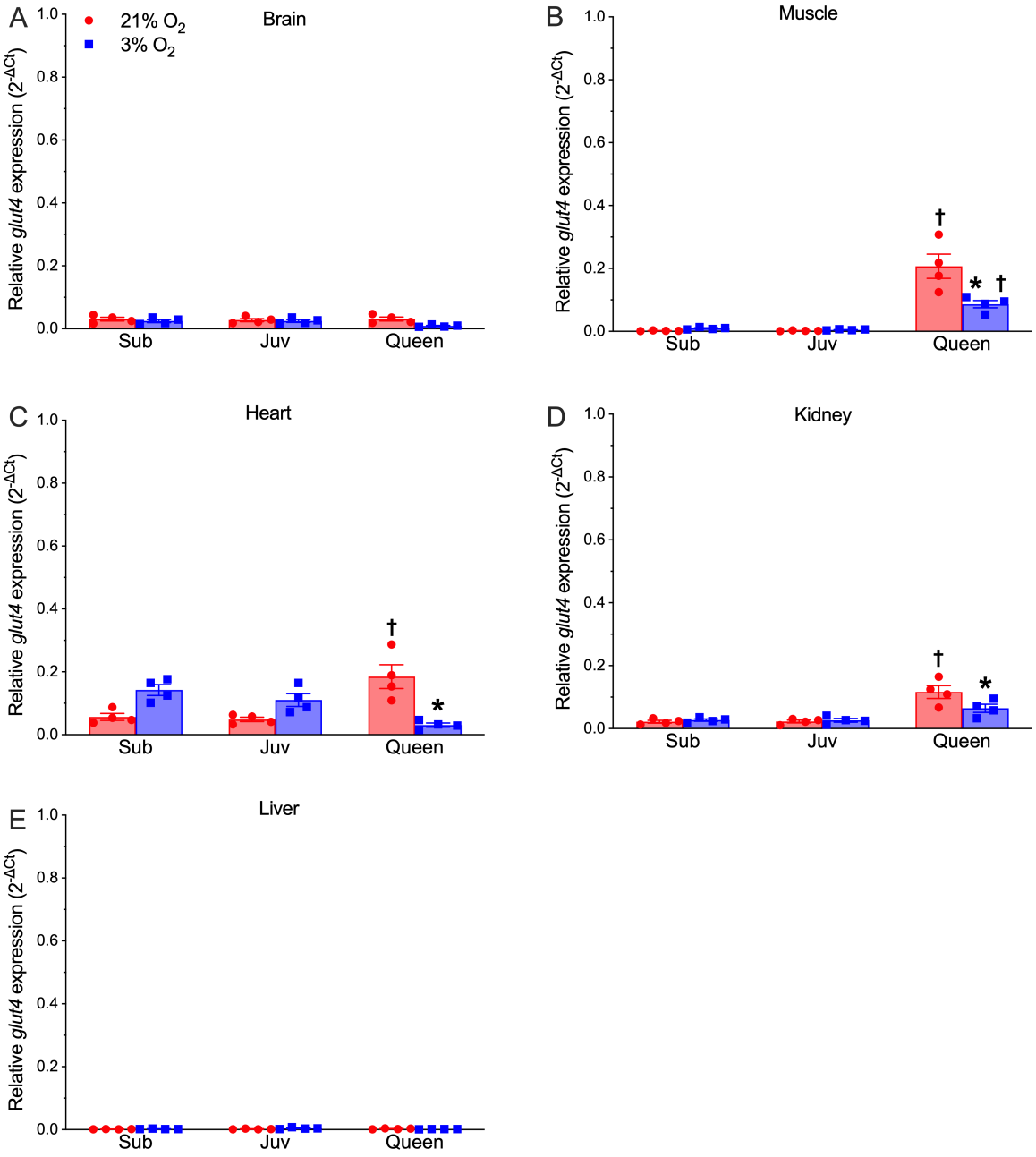


**Figure 4.7 The gene expression of glucose transporter 4 (GLUT4) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. GLUT4 gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

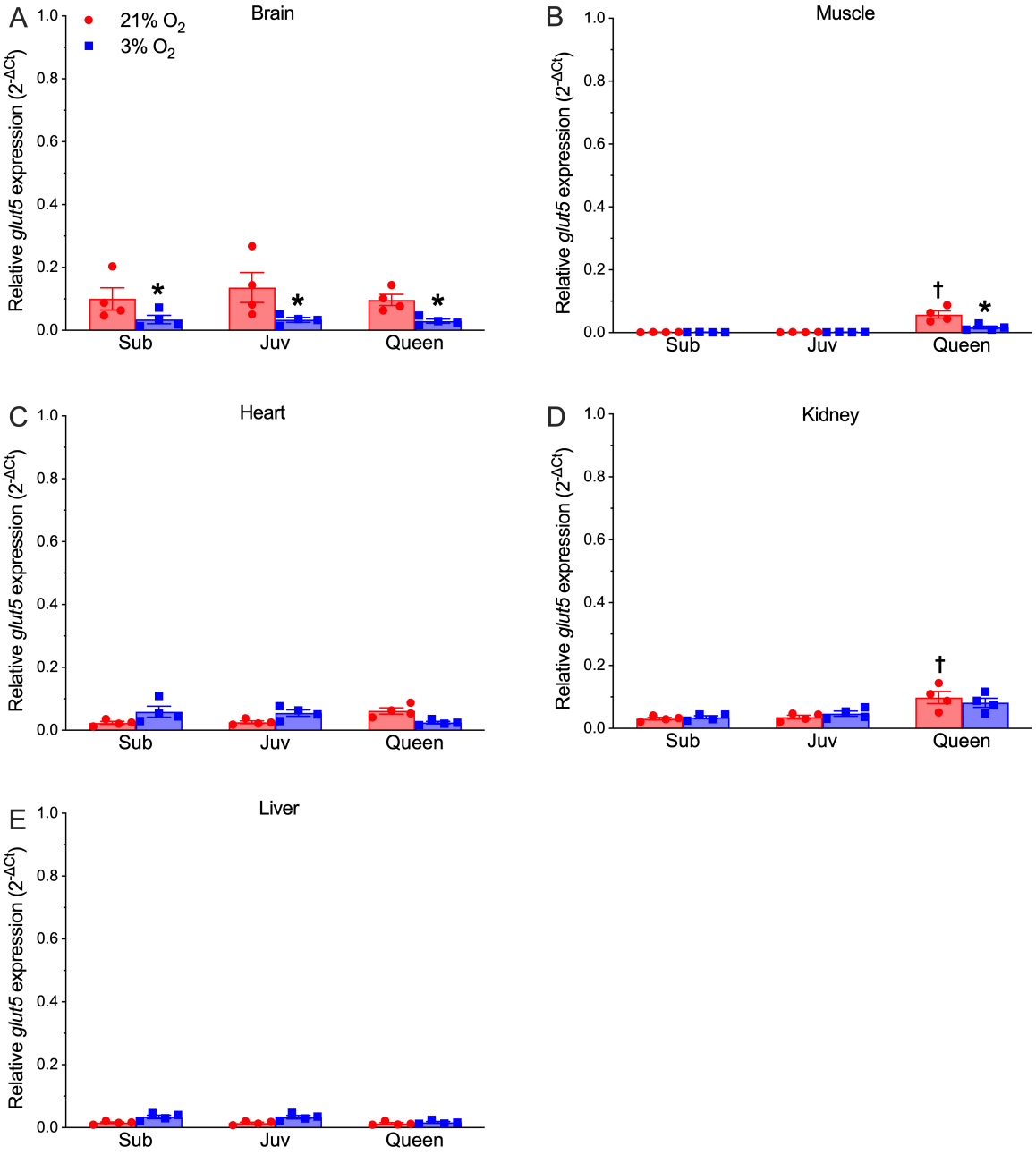


**Figure 4.8 The gene expression of glucose transporter 5 (GLUT5) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. GLUT5 gene expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

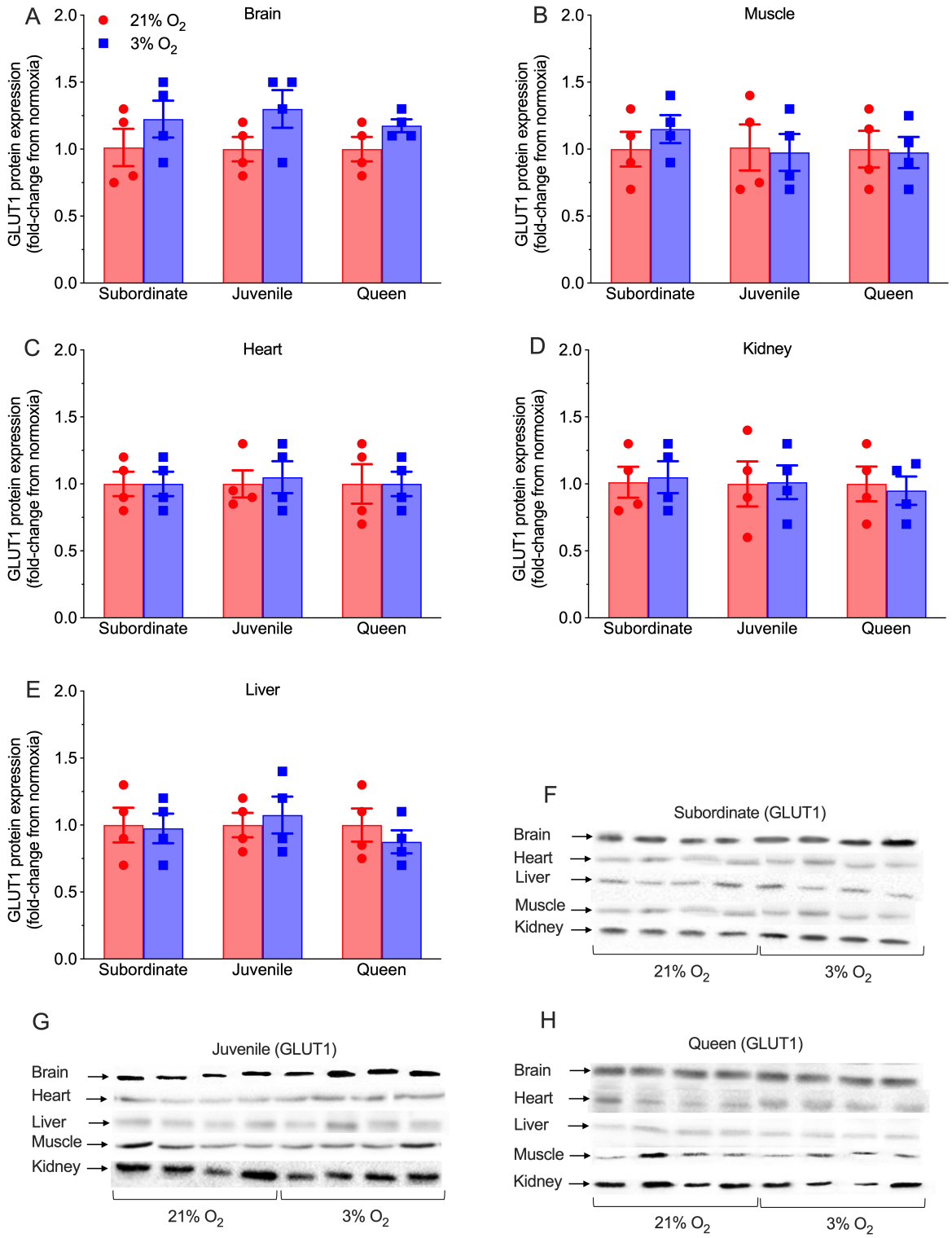


**Figure 4.9 The protein expression of glucose transporter 1 (GLUT1) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. GLUT1 protein expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Panels

(F-H) display blot images from subordinate (F), juvenile (G) and queen (H) animals. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

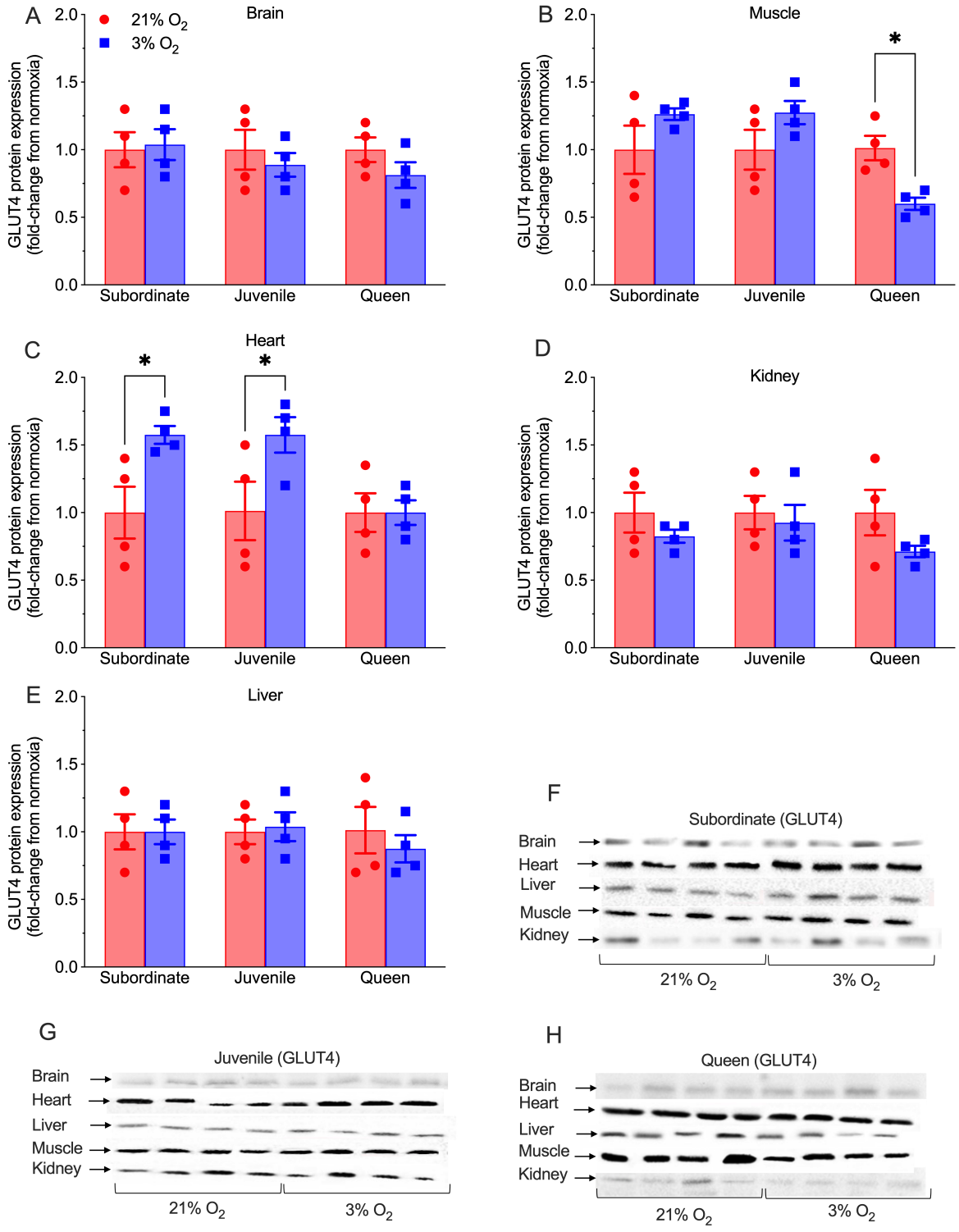

**Figure 4.10 The protein expression of glucose transporter 4 (GLUT4) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. GLUT4 protein expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Panels (F-H) display blot images from subordinate (F), juvenile (G) and queen (H) animals. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste. Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

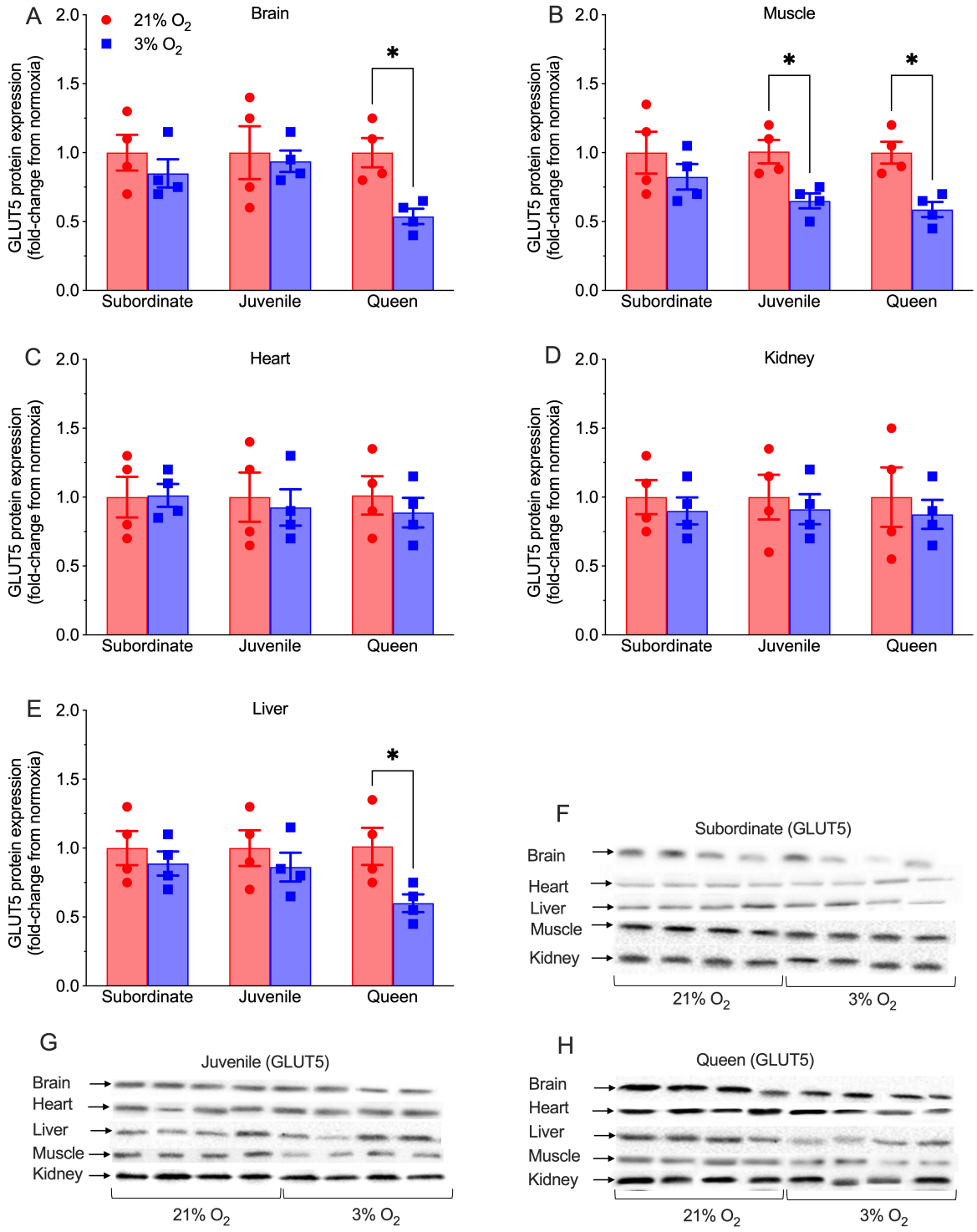


**Figure 4.11 The protein expression of glucose transporter 5 (GLUT5) across tissues and developmental stages in NMRs in normoxia and hypoxia** (n = 4 for each group). Animals were exposed to 21% (red bars) or 3% $O_2$ (blue bars) for 1 hr. GLUT5 protein expression was measured during normoxia and hypoxia in (A) brain, (B) muscle, (C) heart, (D) kidney, and (E) liver. Panels (F-H) display blot images from subordinate (F), juvenile (G) and queen (H) animals. Data are presented as mean ± s.e.m. Asterisks (*) indicate significant effects of hypoxia within each caste.

Daggers (†) indicate significant differences between queens and subordinate animals (two-way ANOVA, $P < 0.05$).

| Gene | Forward Primer (5' to 3') | Reverse Primer (5' to 3') | Efficiency (%) | $R^2$ |
|---|---|---|---|---|
| IGF-1 | CTCTCAATCTTGGCTGCTGGA | GCTGTGGAGCTAGGCGATTAT | 107.2 | 0.98 |
| IGF-2 | GGGAAGGGACTGTGGATGTG | GGTGGACAAGGTATCGCCAA | 98.1 | 0.97 |
| INSR | CGGCAACATCACGCACTAC | CATCCTCAGACTCGAAGGGC | 95.4 | 0.98 |
| IGF1R | AGACACTCAGGACACAAGGC | GCGTAGGGCTGTCTCTCATC | 111.4 | 0.95 |
| IGF2R | TGCCCCATCCAGACAATGAC | TTCCCAATGCCTGAGACCAC | 88.3 | 0.97 |
| GLUT1 | TCCTGCTCATCAACCGCAAT | ATCTGCCGACTCTCCTCCTT | 91.8 | 0.94 |
| GLUT4 | GTCTGAGCACCCCCTCATTC | CAATGCCCTCAGCCTAACCA | 94.3 | 0.97 |
| GLUT5 | GTGCCCCAGCTCTTCATC | GTTCCGAAAACCGAACAGC | 89.4 | 0.93 |
| Eukaryotic elongation factor 2 | CTGCCAGCTCATCCTAGACC | CTTGTCCTTGTCCTCGCTGT | 95.3 | 0.97 |
| Actb | CTCTGTGTGGATCGGTGGC | GGGTGAAAGGCAGCGAAGTA | 97.6 | 0.98 |

**Table 4.1 Primer sequences used for qPCR in this study.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 2.10$, $P = 0.1507$ | $F_{2,18} = 6.16$, $P = 0.0091$ | $F_{1,18} = 18.55$, $P = 0.0004$ |
| Heart | $F_{2,18} = 4.60$, $P = 0.0242$ | $F_{2,18} = 10.63$, $P = 0.0009$ | $F_{1,18} = 9.05$, $P = 0.0075$ |
| Liver | $F_{2,18} = 0.002$, $P = 0.9973$ | $F_{2,18} = 4.43$, $P = 0.0271$ | $F_{1,18} = 3.15$, $P = 0.0928$ |
| Muscle | $F_{2,18} = 3.81$, $P = 0.0415$ | $F_{2,18} = 3.87$, $P = 0.0399$ | $F_{1,18} = 2.44$, $P = 0.1351$ |
| Kidney | $F_{2,18} = 1.56$, $P = 0.2354$ | $F_{2,18} = 13$, $P = 0.0003$ | $F_{1,18} = 1.47$, $P = 0.2399$ |

**Table 4.2 F-statistics analysis of the effects of hypoxia, caste and their interaction on IGF-1 expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on IGF-1 expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on IGF-1 expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 6.05$, $P = 0.0098$ | $F_{2,18} = 0.341$, $P = 0.7152$ | $F_{1,18} = 30.75$, $P < 0.0001$ |
| Heart | $F_{2,18} = 5.71$, $P = 0.0119$ | $F_{2,18} = 7.74$, $P = 0.0037$ | $F_{1,18} = 4.17$, $P = 0.0559$ |
| Liver | $F_{2,18} = 3.54$, $P = 0.0503$ | $F_{2,18} = 15.14$, $P = 0.0001$ | $F_{1,18} = 7.56$, $P = 0.0131$ |
| Muscle | $F_{2,18} = 21.25$, $P < 0.0001$ | $F_{2,18} = 1.05$, $P = 0.3692$ | $F_{1,18} = 0.846$, $P = 0.3698$ |
| Kidney | $F_{2,18} = 5.38$, $P = 0.0147$ | $F_{2,18} = 5.53$, $P = 0.0133$ | $F_{1,18} = 0.261$, $P = 0.6155$ |

**Table 4.3 F-statistics analysis of the effects of hypoxia, caste and their interaction on IGF-2 expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on IGF-2 expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on IGF-2 expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 3.54$, $P = 0.0505$ | $F_{2,18} = 0.724$, $P = 0.4982$ | $F_{1,18} = 48.17$, $P < 0.0001$ |
| Heart | $F_{2,18} = 1.92$, $P = 0.1742$ | $F_{2,18} = 5.60$, $P = 0.0128$ | $F_{1,18} = 75.43$, $P < 0.0001$ |
| Liver | $F_{2,18} = 0.654$, $P = 0.5315$ | $F_{2,18} = 12.91$, $P = 0.0003$ | $F_{1,18} = 0.650$, $P = 0.4303$ |
| Muscle | $F_{2,18} = 6.50$, $P = 0.0075$ | $F_{2,18} = 40.83$, $P < 0.0001$ | $F_{1,18} = 7.61$, $P = 0.0129$ |
| Kidney | $F_{2,18} = 1.24$, $P = 0.3112$ | $F_{2,18} = 18.48$, $P < 0.0001$ | $F_{1,18} = 23.98$, $P = 0.0001$ |

**Table 4.4 F-statistics analysis of the effects of hypoxia, caste and their interaction on insulin receptor expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on insulin receptor expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on IGF-1 expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 5.03$, $P = 0.0183$ | $F_{2,18} = 0.703$, $P = 0.5081$ | $F_{1,18} = 35.28$, $P < 0.0001$ |
| Heart | $F_{2,18} = 13.45$, $P = 0.0003$ | $F_{2,18} = 0.945$, $P = 0.4069$ | $F_{1,18} = 2.16$, $P = 0.1581$ |
| Liver | $F_{2,18} = 1.45$, $P = 0.2591$ | $F_{2,18} = 1.64$, $P = 0.2208$ | $F_{1,18} = 3.78$, $P = 0.0676$ |
| Muscle | $F_{2,18} = 0.36$, $P = 0.7021$ | $F_{2,18} = 15.64$, $P = 0.0001$ | $F_{1,18} = 17.41$, $P = 0.0006$ |
| Kidney | $F_{2,18} = 4.58$, $P = 0.0246$ | $F_{2,18} = 2.72$, $P = 0.0928$ | $F_{1,18} = 3.34$, $P = 0.0842$ |

**Table 4.5 F-statistics analysis of the effects of hypoxia, caste and their interaction on IGF-1 receptor expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on IGF-1 receptor expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on IGF-1 receptor expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 0.55$, $P = 0.5827$ | $F_{2,18} = 10.12$, $P = 0.0011$ | $F_{1,18} = 16.62$, $P = 0.0007$ |
| Heart | $F_{2,18} = 6.26$, $P = 0.0086$ | $F_{2,18} = 15.96$, $P = 0.0001$ | $F_{1,18} = 7.86$, $P = 0.0117$ |
| Liver | $F_{2,18} = 0.04$, $P = 0.9518$ | $F_{2,18} = 5.00$, $P = 0.0187$ | $F_{1,18} = 0.02$, $P = 0.8696$ |
| Muscle | $F_{2,18} = 1.62$, $P = 0.2251$ | $F_{2,18} = 28.72$, $P < 0.0001$ | $F_{1,18} = 6.66$, $P = 0.0188$ |
| Kidney | $F_{2,18} = 0.18$, $P = 0.8289$ | $F_{2,18} = 12.80$, $P = 0.0003$ | $F_{1,18} = 0.55$, $P = 0.4678$ |

**Table 4.6 F-statistics analysis of the effects of hypoxia, caste and their interaction on IGF-2 receptor expression across different tissues.** It presents the results of an F-statistics analysis examining the effect of hypoxia, caste and their interaction on IGF-2 receptor expression in various tissues. The F-values and corresponding P-values indicate the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on IGF-2 receptor expression in the respective tissue**.**

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 0.06$, $P = 0.9361$ | $F_{2,18} = 0.39$, $P = 0.6826$ | $F_{1,18} = 2.71$, $P = 0.1167$ |
| Heart | $F_{2,18} = 7.80$, $P = 0.0036$ | $F_{2,18} = 6.67$, $P = 0.0068$ | $F_{1,18} = 4.71$, $P = 0.0436$ |
| Liver | $F_{2,18} = 3.06$, $P = 0.0718$ | $F_{2,18} = 0.9653$, $P = 0.3997$ | $F_{1,18} = 4.27$, $P = 0.0534$ |
| Muscle | $F_{2,18} = 14.18$, $P = 0.0002$ | $F_{2,18} = 32.52$, $P < 0.0001$ | $F_{1,18} = 1.87$, $P = 0.1880$ |
| Kidney | $F_{2,18} = 6.82$, $P = 0.0062$ | $F_{2,18} = 0.64$, $P = 0.5381$ | $F_{1,18} = 14.00$, $P = 0.0015$ |

**Table 4.7 F-statistics analysis of the effects of hypoxia, caste, and their interaction on GLUT1 mRNA expression across different tissues.** It presents F-values and corresponding P-values, indicating the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on GLUT1 mRNA expression in the respective tissue.

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 0.09$, $P = 0.9139$ | $F_{2,18} = 1.15$, $P = 0.3386$ | $F_{1,18} = 5.25$, $P = 0.0341$ |
| Heart | $F_{2,18} = 0.06$, $P = 0.94$ | $F_{2,18} = 0.02$, $P = 0.97$ | $F_{1,18} = 0.14$, $P = 0.71$ |
| Liver | $F_{2,18} = 0.79$, $P = 0.4674$ | $F_{2,18} = 2.38$, $P = 0.1210$ | $F_{1,18} = 0.03$, $P = 0.8539$ |
| Muscle | $F_{2,18} = 0.40$, $P = 0.6720$ | $F_{2,18} = 0.60$, $P = 0.5594$ | $F_{1,18} = 0.05$, $P = 0.8123$ |
| Kidney | $F_{2,18} = 0.23$, $P = 0.7903$ | $F_{2,18} = 0.53$, $P = 0.5920$ | $F_{1,18} = 0.01$, $P = 0.9120$ |

**Table 4.8 F-statistics analysis of the effects of hypoxia, caste, and their interaction on GLUT1 protein expression across different tissues.** It presents F-values and corresponding P-values, indicating the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on GLUT1 protein expression in the respective tissue.

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 2.08$, $P = 0.1535$ | $F_{2,18} = 1.24$, $P = 0.3127$ | $F_{1,18} = 6.25$, $P = 0.0223$ |
| Heart | $F_{2,18} = 22.06$, $P < 0.0001$ | $F_{2,18} = 1.06$, $P = 0.3640$ | $F_{1,18} = 0.01$, $P = 0.8951$ |
| Liver | $F_{2,18} = 3.07$, $P = 0.0711$ | $F_{2,18} = 1.62$, $P = 0.2253$ | $F_{1,18} = 2.41$, $P = 0.1373$ |
| Muscle | $F_{2,18} = 9.69$, $P = 0.0014$ | $F_{2,18} = 49.35$, $P < 0.0001$ | $F_{1,18} = 7.36$, $P = 0.0142$ |
| Kidney | $F_{2,18} = 4.76$, $P = 0.0219$ | $F_{2,18} = 26.20$, $P < 0.0001$ | $F_{1,18} = 2.84$, $P = 0.1089$ |

**Table 4.9 F-statistics analysis of the effects of hypoxia, caste, and their interaction on GLUT4 mRNA expression across different tissues.** It presents F-values and corresponding P-values, indicating the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on GLUT4 mRNA expression in the respective tissue.

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 0.62$, P = 0.5489 | $F_{2,18} = 0.52$, P = 0.5988 | $F_{1,18} = 0.14$, P = 0.7048 |
| Heart | $F_{2,18} = 3.84$, P = 0.0408 | $F_{2,18} = 0.07$, P = 0.9285 | $F_{1,18} = 7.19$, P = 0.0152 |
| Liver | $F_{2,18} = 2.63$, P = 0.0992 | $F_{2,18} = 0.15$, P = 0.8588 | $F_{1,18} = 0.75$, P = 0.3961 |
| Muscle | $F_{2,18} = 4.88$, P = 0.0203 | $F_{2,18} = 4.07$, P = 0.0347 | $F_{1,18} = 0.13$, P = 0.7183 |
| Kidney | $F_{2,18} = 0.30$, P = 0.7381 | $F_{2,18} = 2.29$, P = 0.1296 | $F_{1,18} = 1.10$, P = 0.3075 |

**Table 4.10 F-statistics analysis of the effects of hypoxia, caste, and their interaction on GLUT4 protein expression across different tissues.** It presents F-values and corresponding P-values, indicating the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on GLUT4 protein expression in the respective tissue.

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 0.31$, P = 0.7332 | $F_{2,18} = 0.39$, P = 0.6804 | $F_{1,18} = 13.46$, P = 0.0018 |
| Heart | $F_{2,18} = 8.55$, P = 0.0024 | $F_{2,18} = 0.03$, P = 0.9629 | $F_{1,18} = 1.36$, P = 0.2587 |
| Liver | $F_{2,18} = 2.56$, P = 0.1044 | $F_{2,18} = 4.12$, P = 0.0335 | $F_{1,18} = 17.89$, P = 0.0005 |
| Muscle | $F_{2,18} = 10.97$, P = 0.0008 | $F_{2,18} = 33.05$, P < 0.0001 | $F_{1,18} = 11.17$, P = 0.0036 |
| Kidney | $F_{2,18} = 0.86$, P = 0.4379 | $F_{2,18} = 15.17$, P = 0.0001 | $F_{1,18} = 0.0003$, P = 0.9855 |

**Table 4.11 F-statistics analysis of the effects of hypoxia, caste, and their interaction on GLUT5 mRNA expression across different tissues.** It presents F-values and corresponding P-values, indicating the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on GLUT5 mRNA expression in the respective tissue.

| Tissue | Interaction | Effect of caste | Effect of hypoxia |
|---|---|---|---|
| Brain | $F_{2,18} = 1.22$, P = 0.3171 | $F_{2,18} = 4.07$, P = 0.0347 | $F_{1,18} = 7.5$, P = 0.0135 |
| Heart | $F_{2,18} = 1.67$, P = 0.2159 | $F_{2,18} = 1.04$, P = 0.3728 | $F_{1,18} = 0.51$, P = 0.4825 |
| Liver | $F_{2,18} = 1.68$, P = 0.2127 | $F_{2,18} = 1.37$, P = 0.2794 | $F_{1,18} = 6.12$, P = 0.0235 |
| Muscle | $F_{2,18} = 2.29$, P = 0.1293 | $F_{2,18} = 7.01$, P = 0.0056 | $F_{1,18} = 19.09$, P < 0.0004 |
| Kidney | $F_{2,18} = 0.50$, P = 0.6147 | $F_{2,18} = 0.79$, P = 0.4688 | $F_{1,18} = 4.12$, P = 0.0572 |

**Table 4.12 F-statistics analysis of the effects of hypoxia, caste, and their interaction on GLUT5 protein expression across different tissues.** It presents F-values and corresponding P-values, indicating the statistical significance of each factor. Significant effects ($P < 0.05$) suggest a notable impact on GLUT5 protein expression in the respective tissue.

## 4.5 Discussion

This study provides new insights into the molecular mechanisms by which NMRs adapt to severe acute hypoxia, revealing distinct transcriptional and translational strategies across developmental stages and social castes. Specifically, we examined how the expression of IGFs, their receptors, and glucose and fructose transporters are regulated in response to hypoxic stress. Consistent with our prior findings of elevated blood glucose and lactate levels under hypoxia (Chapters 2 and 3), our current data demonstrate that these systemic changes are accompanied by complex, tissue-specific molecular responses. Notably, while queens generally exhibited stable or downregulated expression profiles across most tissues, subordinates and juveniles showed largely similar patterns of unchanged or decreased IGF and glucose transporter expression, except in the heart, where IGF and GLUT4 expression tended to increase. Additionally, there are significant differences in baseline expression levels across both normoxic and hypoxic conditions between subordinates and queens in several tissues, particularly in the brain, heart, and muscle, suggesting intrinsic caste-based regulatory differences. These differences suggest that developmental stage influences the magnitude and tissue specificity of molecular responses. These results partially contradict our initial hypothesis of uniform IGF suppression and transporter downregulation, instead revealing that caste- and stage-specific strategies are employed to meet differing physiological demands.

This divergent molecular phenotype likely reflects the distinct roles within the colony, subordinates and juveniles engage in energetically demanding activities such as foraging and burrowing, while queens maintain a more sedentary lifestyle focused on reproduction. Collectively, these results highlight how metabolic flexibility in NMRs is shaped by both social hierarchy and developmental context to support survival under $O_2$-limited conditions.

***Tissues specific changes in the expression of IGF, their receptors and insulin receptor***. Our study demonstrates a marked suppression of IGF signaling in the brain under hypoxic conditions, particularly in subordinate and juvenile NMRs. IGF-1 expression was significantly decreased in subordinates, with a decreasing trend observed in juveniles and queens. IGF1R levels were reduced in both subordinates and juveniles, with a similar trend noted in queens. Similarly, IGF-2 expression was downregulated in subordinates and juveniles, with a downward trend in queens; however, IGF2R expression showed a non-significant decrease across all groups. These coordinated reductions suggest a robust hypoxia-driven regulatory mechanism. Notably, IGF1R suppression was consistent across the two non-reproductive castes, supporting the idea that IGF signaling may play an important role in regulating blood glucose in subordinates and juveniles, as described in chapter 2. These findings align with our previous data in chapter 3, which demonstrated a general reduction in glycolytic enzyme activity in the brain and are consistent with prior research on metabolic suppression in the NMR brain during hypoxia (156, 184). Interestingly, under normoxic conditions, IGF-1 expression in queens was significantly lower than in subordinates or juveniles, while no significant differences were observed between castes under hypoxia. This suggests that subordinates and juveniles may suppress glucose uptake under hypoxic conditions to conserve energy and rely more heavily on local carbohydrate utilization. This adaptation may represent a strategic shift in energy metabolism tailored to $O_2$ availability. Collectively, these data support the hypothesis that the downregulation of IGFs and their receptors serves as a protective mechanism, potentially mitigating lactate-induced acidosis and its harmful effects on neurons.

Beyond its metabolic effects, suppression of IGF1R may also contribute to long-term health span and stress resilience. . IGF1R activation supports the PI3K/Akt pathway, which

regulates cell growth and development (218). Studies in various species suggest that reduced IGF1R activity extends lifespan, whereas excessive activation promotes tumorigenesis (179, 219, 220). Additionally, IGF1R heterozygous knockout in mice enhances oxidative stress resistance and longevity (220). Given the remarkable lifespan and cancer resistance of NMRs, the brain-specific suppression of IGF1R under hypoxia may contribute to these traits by limiting anabolic activity and reducing cellular stress.

Likewise, IGF2R downregulation may help regulate IGF-2 levels. Unlike IGF1R, IGF2R does not activate the PI3K/Akt pathway but primarily functions as a clearance receptor (221-223). Its reduced expression could slow IGF-2 degradation, allowing controlled bioavailability while reducing the risk of excessive mitogenic signaling which has been associated with uncontrolled cell proliferation (224, 225). This modulation may contribute to metabolic suppression, a concept introduced earlier, by limiting cell proliferation and conserving energy under hypoxic conditions (226). Additionally, queens under hypoxia exhibited higher expression levels than subordinates and juveniles. This elevated expression may reflect caste-specific regulation. Increased IGF2R levels in queens could enhance IGF-2 clearance, helping to fine-tune local growth factor signaling and prevent excessive mitogenic activity, consistent with cancer resistance and metabolic restraint in this caste proliferation (224, 225).

The decrease in insulin receptor expression indicates a metabolic shift away from insulin-dependent glucose metabolism. As a key regulator of glucose homeostasis, insulin receptor suppression suggests an adaptive strategy to modify energy utilization under low $O_2$. This downregulation may reduce glucose uptake and anabolic processes, favoring alternative pathways to sustain cellular function during hypoxia (227, 228).

Conversely, tissues such as the heart and muscle exhibit adaptive shifts in IGF regulation. In the heart, IGF expression trends upward, while IGF receptor levels remain stable and insulin receptor expression declines by 90%. This shift suggests a potential reliance on IGF signaling as an alternative metabolic pathway, ensuring sustained glycolysis despite reduced insulin activity. Similarly, in muscle, IGF expression shows a general increase under hypoxia, potentially serving as a localized mechanism to support cellular energy homeostasis in the face of systemic IGF suppression.

In contrast, the liver and kidney maintain relatively stable IGF expression, with the liver exhibiting the highest IGF levels among all tissues. This stability indicates the central role of the liver in systemic IGF production, ensuring endocrine support despite environmental stress. The kidney, although showing unchanged IGF expression, exhibits a notable 66% decrease in insulin receptor levels and a non-significant increase in IGF1R expression. This pattern may suggest a shift toward IGF-driven metabolic regulation, enabling the kidney to sustain essential functions despite systemic metabolic constraints.

Furthermore, increased IGF signaling may also stimulate gluconeogenesis in the liver (215, 227), ensuring a steady supply of glucose to vital organs, such as the brain and heart. This dual role of promoting glycolysis and gluconeogenesis highlights the central role of the liver in systemic adaptation to hypoxia.

The increased IGF expression may have protective effects, mitigating hypoxia-induced cellular damage (229, 230). IGF signaling is known to activate survival pathways, such as the PI3K/Akt pathway, which reduces apoptosis and promotes cell repair. Additionally, IGF can enhance antioxidant defenses by upregulating enzymes that counteract oxidative stress, further protecting cells from hypoxic injury (231).

Interestingly, while the expression of IGF and all three receptors remained unchanged in most tissues of queens, it showed a general downward trend. Meanwhile, hepatic mRNA levels exhibited minimal fluctuation, remaining stable under hypoxia. This stability suggests the liver maintains a critical metabolic role in preserving glucose availability for queens during hypoxia. The differences in IGF expression between subordinates/juveniles and queens highlight distinct adaptive strategies within the colony. Subordinates and juveniles enhance glucose uptake and glycolysis, likely to meet the immediate energy demands during hypoxia. In contrast, queens exhibit widespread downregulation of IGF expression, aligning with an energy-conserving strategy. This shift may involve reliance on alternative pathways, such as fatty acid oxidation, to sustain metabolic functions while minimizing energy expenditure.

Previous studies on the IGF system in NMRs indicate a high degree of similarity to humans and mice, but the regulatory mechanisms in NMRs are uniquely adapted (179). Variations in IGF binding proteins (IGFBPs) and the protease pregnancy-associated plasma protein-A (PAPP-A) likely modulate IGF bioavailability and signaling, contributing to their exceptional traits, such as extended longevity and cancer resistance. These interspecies differences, along with variations across developmental stages and hierarchical statuses within NMR colonies, highlight the complexity of IGF regulation in this species.

***Discrepancies between mRNA and protein expression of GLUTs***. Our results reveal a clear discrepancy between the transcriptional and translational regulation of GLUT1, GLUT4, and GLUT5 in subordinates, juveniles, and queens during hypoxia. At the gene expression level, hypoxia increases GLUT1 expression in the heart and kidney of subordinates and the muscle of queens; however, protein levels of GLUT1 remained unchanged across all groups. Regarding

GLUT4, gene expression decreases in the heart, muscle and kidney of queens, while at the protein level, a reduction is observed only in the muscle of queens and the heart of subordinates and juveniles. For GLUT5, gene expression decreases across all groups in the brain and also in the muscle of queens. However, at the protein, GLUT5 shows a decrease in the brain, liver and muscle of queens, as well as in the muscle of juveniles. This difference between gene and protein expression could be attributed to the time-dependent nature of translational processes, as protein synthesis is not instantaneous. The rapid increase in GLUT4 in the heart may be explained by tissue-specific mechanisms, such as the mobilization of pre-existing intracellular GLUT4 vesicles in the heart or enhanced translation efficiency in these critical tissues, allowing them to maintain glucose uptake before newly synthesized proteins accumulate (232, 233).

This selective increase at the protein level in the heart underscores their crucial roles in maintaining cardiac function during hypoxia. Notably, the upregulation of GLUT4 in the heart suggests an adaptive mechanism to sustain cardiac performance under hypoxic stress.

Previous studies indicate that GLUT1 and GLUT4 undergo significant regulatory changes during hypoxia to enhance glucose uptake in hypoxic tissues. GLUT1, responsible for basal glucose transport, is rapidly upregulated at both transcriptional and protein levels in response to hypoxia, primarily through the activation of hypoxia-inducible factor 1, facilitating increased glucose uptake under low $O_2$ conditions (232, 234, 235). In contrast, GLUT4, an insulin-responsive glucose transporter, responds to hypoxia through rapid translocation from intracellular vesicles to the plasma membrane, a process mediated by AMP-activated protein kinase activation. This translocation enhances glucose uptake, particularly in skeletal muscle cells, during hypoxic conditions (236, 237). These complementary mechanisms ensure that tissues maintain adequate glucose uptake during hypoxic stress.

Our findings show that GLUT1 and GLUT4 expression in skeletal muscle remain unchanged following 1 hr of 3% $O_2$ in subordinates and juveniles. This lack of change in GLUT4 expression in our study contrasts with findings in hypoxia-intolerant mice exposed to 45 min of 1% $O_2$ (238) and in NMRs subjected to 4 hr of 3% $O_2$ (167). In mice, hypoxia activates AMPK, leading to increased GLUT4 expression in skeletal muscle and enhanced carbohydrate uptake into cells. Conversely, studies on skeletal muscle in NMRs during 4 hr of 3% $O_2$ revealed that protein expression of GLUT1 and ACC1 remained unchanged, while GLUT4 and p-GS were downregulated. This suggests that NMRs may reduce glucose uptake in skeletal muscle over longer hypoxic periods while continuing to metabolize lipids to meet energy demands.

Furthermore, 1 hr of hypoxia might not be sufficient to induce transcriptional changes or protein production required to alter glucose transporter expression significantly. Instead, this shorter exposure may reflect an initial adaptive phase where energy demands are met without substantial molecular remodeling, relying on existing protein pools and metabolic flexibility.

In our study, GLUT5 protein expression in the heart and brain of subordinates and juveniles remained unchanged, while a previous study reported its upregulation in anoxic conditions within 10-30 mins (122). Additionally, previous studies reported that GLUT5 expression in the brain decreased under milder hypoxia (7% $O_2$ for 4 hr) (127), indicating a potential temporal and $O_2$-level-dependent regulatory mechanism.

In stark contrast, queens displayed a general downregulation trend of GLUT1, GLUT4, and GLUT5 expression across most tissues or unchanged in some of them at both the gene and protein levels. This downregulation indicates a shift towards a more energy-conservative state, where queens likely reduce their dependency on glucose uptake and utilization to preserve energy reserves and minimize metabolic demands. The reduction in both gene and protein expression in

most tissues suggests that queens prioritize long-term survival and metabolic stability over short-term energy production in hypoxic environments. This strategy might reflect the role of queens in colony stability and reproduction, where energy conservation becomes paramount during prolonged environmental stress.

These findings highlight significant developmental and social status-dependent differences in glucose transporter regulation among NMRs. Subordinates and juveniles appear to employ a targeted approach, with expression remaining unchanged across most tissues, which may support the maintenance of essential pathways for immediate survival and function during acute hypoxia. Conversely, queens minimize resource depletion through a metabolic strategy that could sustain colony resilience during chronic or repeated hypoxic exposure. It remains to be determined whether this queen-specific metabolic shift reflect a general reproductive effect, potentially influenced by hormonal factors such as estrogen or prolactin, or represents a specialized adaptation unique to NMRs. Given the central role of queens in reproduction and colony stability, future studies examining endocrine influences on energy regulation during hypoxia may provide further insights.

## 5 General conclusions

Hypoxia is a critical driver of numerous pathological conditions, including stroke, obesity, neurodegenerative diseases, and cancer (239-241). Central to survival under low $O_2$ conditions is the regulation of cellular energy metabolism, particularly glucose metabolism, which serves as a key mechanism to meet ATP demands in hypoxic environments (242). A central challenge lies in elucidating how cells transition from utilizing diverse fuel substrates in normoxia to prioritizing specific metabolic pathways in hypoxia to sustain energy production.

NMRs represent an excellent model for studying mechanisms of hypoxia tolerance in mammals. Under laboratory conditions, they can tolerate prolonged exposure to 3% $O_2$ (155, 188, 243) and even transient anoxia (122) without incurring significant cellular damage. Mounting evidence suggests that NMRs employ a suite of adaptive mechanisms, with glucose metabolism playing a pivotal role in both initiating and sustaining these responses.

In this thesis, I investigate glucose metabolism as a central regulatory axis for hypoxia adaptation across five key organs, brain, heart, liver, kidney, and skeletal muscle, and at distinct developmental stages of NMRs, spanning pups, juveniles, subordinate, and queen adults. By integrating developmental and social hierarchy perspectives, this work examines how glucose utilization strategies evolve from early life to adulthood and how they diverge between non-reproductive subordinates and the queen, a fascinating model for studying metabolic trade-offs linked to reproductive status and hypoxia resilience.

My findings reveal tissue-specific mechanisms of hypoxia adaptation, including enhanced anaerobic glycolysis, redox balance maintenance, and metabolic flexibility to prioritize ATP-efficient pathways. Notably, this multi-tissue, developmental-stage analysis uncovers both conserved and divergent regulatory strategies in glucose metabolism across colony members,

highlighting the interplay between individual variation and collective survival in social species like NMRs.

***Metabolic suppression in the NMR brain.*** Previous studies on subordinate NMRs showed that hypoxia induces profound metabolic suppression in their brains (156, 158, 184). In this study, we also demonstrate that all developmental stages of NMRs exhibit decreased gene and protein expression in the brain under acute severe hypoxia (3% $O_2$), whereas these changes are minimal or absent in queens. These changes in subordinates and juveniles are characterized by a 75% reduction in IGF and insulin receptor expression and a 90% decline in IGF levels, whereas they are less pronounced in queens (Chapter 4). This suppression extends to glycogenolysis, as evidenced by a significant downregulation of glycogen phosphorylase and lactate export via MCT4 expression (Chapter 3). Notably, GLUT1 protein expression exhibits a transient upregulation in subordinates and juveniles, ensuring continued glucose uptake to support essential neuronal functions during acute hypoxia. In contrast, queens show no change in GLUT1 expression, reflecting their prioritization of long-term energy conservation over transient metabolic activity (Chapter 4).

This ability to dynamically regulate metabolism under $O_2$ deprivation may help the NMR brain tolerate hypoxia without cellular dysfunction. For example, unlike in stroke, where reduced blood flow leads to nutrient shortages and energy collapse, NMRs suppress cellular biochemical activity to levels where diminished blood flow does not cause deficiencies, making them uniquely adapted to extreme environmental conditions (244).

NMRs employ a multi-tiered neuroprotective strategy, with metabolic suppression as the primary adaptation enabling survival under hypoxia. This is reinforced by mitochondrial

resilience, redox stability, and the activation of protective proteins, which together prevent neuronal damage that typically occurs in hypoxia-intolerant species. A core component of this adaptation is metabolic reprogramming, which allows NMR brains to suppress ATP-consuming processes while maintaining essential function. Studies showed that NMRs exhibit partial activation of the pentose phosphate pathway during hypoxia (185) and utilize fructose metabolism in anoxia (122) to support glucose metabolism. Additionally, anabolic pathways (e.g., phenylalanine, tyrosine, and tryptophan synthesis) are downregulated, minimizing unnecessary energy expenditure (185). These metabolic shifts may reduce ATP demand, contributing to cellular resilience and energy conservation under hypoxia.

Mitochondrial adaptation enhances metabolic suppression, making NMR brains resistant to ischemic damage (245). Studies show that NMR mitochondria maintain respiratory capacity at a lower rate during ischemia, optimizing ATP production with minimal $O_2$ consumption through enhanced coupling efficiency and reduced electron leakage (245). This adaptation protects against oxidative damage by limiting free radical generation and stabilizing redox balance. Unlike mice, NMR brains preserve mitochondrial integrity and experience minimal cell death post-ischemia, indicating a controlled metabolic slowdown rather than energy collapse (245), allowing them to tolerate ischemic stress similarly to their well-documented hypoxia resistance (156).

Studies also show that HIF-1α expression increases in the NMR brain during hypoxia (7% $O_2$ for 4 hr), triggering cell survival pathways, metabolic suppression, and anti-apoptotic mechanisms to ensure brain protection. Specifically, HIF-1α upregulates NFκB1, a factor that supports anti-apoptotic and oxidative stress responses. Additionally, the activation of p21/CIP1 induces cell-cycle arrest, conserving energy, while the suppression of CYC and JNK reduces mitochondrial-driven apoptosis (246).

The NMR brain suppresses metabolism under hypoxia (7% $O_2$ for 24 hr), and exhibits reduced oxidative stress, minimal DNA/RNA damage, and enhanced antioxidant capacity (184). These adaptations suggest a protective strategy that preserves energy while preventing hypoxia-induced damage.

Studies show that mitochondrial $Ca^{2+}$ buffering in NMR brains increases compared with mice during hypoxia, which supports metabolic suppression by reducing the energy required for $Ca^{2+}$ regulation, preserving ATP, and preventing mitochondrial dysfunction (247). Enhanced $Ca^{2+}$ sequestration in NMR mitochondria minimizes the need for active $Ca^{2+}$ extrusion, stabilizes oxidative phosphorylation, and lowers oxidative stress, further supporting energy conservation. Additionally, these adaptations may contribute to synaptic downregulation, reducing neuronal excitability and overall metabolic demand, ensuring long-term neuronal survival under $O_2$-limited conditions (247).

Taken together, these findings highlight a multi-tiered neuroprotective strategy in NMRs, integrating metabolic suppression, mitochondrial resilience, selective activation of survival pathways, and precise control over redox and calcium dynamics. These adaptations position NMRs as a valuable model for developing therapeutic strategies against ischemic stroke and other hypoxia-related neuropathologies.

***Metabolic adaptation in the NMR heart.*** Our results indicate that PK and LDH activity remains stable across all groups, with an increase in PK observed in subordinates, suggesting that glycolysis is maintained at a steady rate to meet energy demands (Chapter 3). Notably, subordinates and juveniles exhibit a hypoxia-driven upregulation of GLUT4 in the heart, reflecting an increased reliance on glucose uptake to meet energy demands during $O_2$ scarcity (Chapter 4).

This aligns with their broader metabolic shift toward carbohydrate utilization in hypoxia (Chapter 2). Paradoxically, while IGF expression is upregulated in these groups, their insulin receptors are downregulated, a pattern that would typically predict reduced GLUTs activity. However, the observed increase in GLUT4 protein expression suggests compensatory mechanisms to sustain glucose uptake, highlighting the complexity of hypoxia-responsive regulation. In contrast, queens display marked downregulation of GLUT5 (Chapter 4), indicative of a metabolic strategy to limit fructose uptake and prioritize long-term energy conservation over immediate ATP generation, consistent with their focus on metabolic stability.

Faulkes et al. demonstrated that NMR hearts store exceptionally high levels of glycogen, surpassing even the glycogen content found in the mouse liver (187). This glycogen reserve serves as a critical energy buffer. This suggests that queens may primarily sustain ATP production using heart glycogen without actively increasing GLUT4-mediated glucose uptake, while subordinates and juveniles might utilize both heart glycogen and blood glucose as energy sources.

Our study also shows that MCT4 expression remained unchanged across all groups, despite a 75% increase in subordinates and juveniles and a 46% decrease in queens, as these changes did not reach statistical significance (Chapter 3). Previous studies indicate that NMR hearts accumulate lactate intracellularly during ischemia rather than effluxing it (248). This pattern aligns with our findings in subordinates and juveniles, where they increased GLUT4 expression and glycolytic enzyme activity, while unchanged MCT4 levels resulted in intracellular lactate accumulation.

Additionally, unlike most mammals, NMRs decrease their heart rate in extreme hypoxia, while cardiac output remains stable or even increases relative to systemic metabolic depression (123, 157). Studies also suggest that cellular metabolic depression may not be fully activated in

the NMR heart (249, 250). This further supports the idea that the heart continues to meet its ATP demands during hypoxia, ensuring sustained cardiac function in low-$O_2$ conditions.

Another metabolic adaptation that may enhance NMR cardiac function in hypoxia is its ability to rely on fructose metabolism. NMRs metabolize fructose *ex vivo*, demonstrating their capacity to use this alternative fuel (122). This adaptation enables cells to continue generating ATP and sustaining glycolytic flux even when $O_2$ availability is limited. Studies showed that NMR hearts upregulate GLUT5 expression in anoxia, facilitating fructose transport across the membrane (122). However, in our study, GLUT5 expression remained unchanged in subordinates and juveniles during hypoxia, while it decreased in queens. Notably, unlike in other mammals, where fructose metabolism is associated with disease, NMRs maintain cardiac function and cancer resistance into old age (13, 251), allowing them to utilize this metabolic shift without adverse effects.

Studies show that HIF-1α may facilitate metabolic adaptation in the NMR heart, enabling efficient regulation under hypoxic conditions (248). For example, unlike other mammals, where HIF-1α is rapidly degraded in normoxia and activated only under low $O_2$ conditions (252), NMRs maintain its stabilization even in normoxia (248). This constitutive activation serves as a preemptive adaptation, ensuring that glycolysis and glucose uptake remain continuously upregulated. As a result, NMRs can rapidly adjust to fluctuating $O_2$ availability without experiencing metabolic instability or energy depletion (248). This adaptation is particularly critical in protecting cardiac tissue from ischemic injury.

Taken together, these findings suggest that NMRs sustain cardiac function in extreme hypoxia through distinct metabolic strategies. Subordinates and juveniles enhance glucose uptake via GLUT4 and potentially utilize both glycogen and blood glucose to meet energy demands, while

queens prioritize metabolic stability by downregulating GLUT5 and likely relying primarily on glycogen reserves for sustained ATP production.

***Metabolic adaptation in the NMR liver.*** The liver plays a central role in glucose homeostasis during hypoxia in NMRs, with caste-dependent metabolic strategies. Subordinates prioritize glucose mobilization, while queens maintain stability, mobilizing glucose through alternative pathways rather than glycogen breakdown (Chapters 3&4).

Our study shows that subordinates and juveniles increase their blood glucose levels during hypoxia, accompanied by an increase in glycogenolysis enzyme expression in the liver under hypoxia (Chapters 2&3), indicating active glycogen breakdown. Additionally, the upregulation of G6Pase in subordinates (~80% increase) and juveniles (~60% increase) enhances hepatic glucose production (Chapter 3). In contrast, queens elevate blood glucose levels in hypoxia without increasing glycogenolysis enzyme expression (Chapters 2&3). Instead, they decrease PCK and G6Pase enzyme expression in hypoxia while also exhibiting a general reduction in hepatic glucose transporter expression, suggesting decreased glucose uptake by the liver during hypoxia (Chapters 3&4). Taken together, our findings indicate that subordinates rely on glycogen breakdown and gluconeogenesis for immediate energy, while queens maintain blood glucose levels without depleting liver glycogen. Queens likely achieve this by reducing hepatic glucose uptake and limiting peripheral glucose use. This dual strategy ensures that subordinates meet the energy demands of hypoxia, while queens preserve glucose for survival and reproduction.

Previous studies in the NMR liver show that glucose-6-phosphate, fructose-6-phosphate, and glucose-1-phosphate are significantly reduced under hypoxia (7% $O_2$ for 4 hr) (185). Since glucose-6-phosphate, fructose-6-phosphate are key intermediates in glycolysis, their decline

suggests increased glycolytic flux, indicating a shift in glucose utilization during hypoxia (33). They also reported that NMRs accumulate alanine in hypoxia, suggesting suppressed gluconeogenesis (185) as alanine is a key gluconeogenic substrate that is normally converted to glucose via the glucose-alanine cycle (33).

Taken together, these findings indicate that glucose metabolism varies across different hypoxia levels, durations, and developmental stages. The liver may adopt alternative metabolic strategies, potentially enhancing gluconeogenesis, altering glucose utilization, or shifting toward alternative energy substrates such as fatty acids or ketone bodies. Further investigation is needed to determine whether the severity and duration of hypoxia dictate distinct regulatory mechanisms governing hepatic glucose metabolism.

***Metabolic adaptation in the NMR muscle.*** Muscle metabolism in NMRs exhibits a caste-specific response to hypoxia, enabling subordinates and juveniles to sustain energy production, while queens prioritize systemic glucose conservation (Chapters 3&4). IGF hormone expression and receptor levels remain stable in subordinates and juveniles but decrease in queens (Chapter 4), suggesting that younger and working individuals maintain growth and energy signaling, whereas queens suppress it to minimize energy expenditure. Similarly, glucose transporters remain unchanged in subordinates and juveniles but decrease in queens, further restricting glucose uptake in reproductive individuals (Chapter 4).

To sustain ATP production under hypoxia, subordinates and juveniles enhance glycolytic activity, lactate production, and lactate export for anaerobic metabolism while, queens downregulate these pathways, reinforcing their strategy of glucose conservation over rapid energy use (Chapter 3).

Glycogen metabolism follows a similar pattern, with subordinates and juveniles maintain stable PHKG2 and PYGM expression, ensuring glycogen availability for energy use when needed. In contrast, queens downregulate PHKG2 and PYGM, further limiting glycogen breakdown and reinforcing their shift away from muscle-based carbohydrate metabolism (Chapter 3). This metabolic divergence may be linked to differences in fuel substrate preference (Chapter 2); under normoxia, subordinates and juveniles rely on lipids for energy but switch to carbohydrates in hypoxia, maintaining glycogen breakdown to meet ATP demands. In contrast, queens consistently use carbohydrates in all conditions, leading to suppressed glycogen breakdown under hypoxia, prioritizing systemic glucose stability over immediate energy release.

Previous studies show that NMR mitochondria maintain a stable metabolic function in skeletal muscle, likely supporting consistent movement between normoxic and hypoxic environments with minimal energetic costs (186). Specifically, lipid-fueled respiration (palmitoylcarnitine-malate) decreased by ~ 15% during both acute (7% $O_2$ for 4 hr) and chronic hypoxia (11% $O_2$ for 4–6 weeks), indicating a slight suppression of lipid metabolism under low $O_2$ conditions. However, carbohydrate-fueled respiration (pyruvate-malate) remained unchanged (186), suggesting that skeletal muscle did not shift towards greater carbohydrate dependence, unlike other hypoxia-tolerant species (253). Notably, this metabolic stability is accompanied by stable ROS levels, maintained through enhanced mitochondrial antioxidant defenses (254), further supporting the idea that NMRs adopt a moderate but effective evolutionary strategy to cope with fluctuating $O_2$ availability.

***Metabolic adaptation in the NMR kidney.*** Kidney metabolism remains stable in subordinates and juveniles, while queens suppress certain pathways to conserve energy (Chapters 3&4). IGF

hormones expression remained unchanged across all groups, but queens downregulate insulin receptors, reducing their sensitivity to IGF signaling (Chapter 4). Similarly, glucose transporters remain stable in subordinates and juveniles but decrease in queens (Chapter 4), limiting glucose uptake to preserve systemic energy. In addition, glycolytic enzyme activity increases in subordinates and juveniles, but remains unchanged in queens (Chapter 3), reinforcing their differing metabolic strategies. These findings highlight a caste-specific adaptation in kidney metabolism, where subordinates and juveniles sustain glucose transport and lactate clearance, while queens restrict IGF signaling and glucose uptake, reinforcing their energy conservation strategy. This mirrors broader metabolic trends across tissues, ensuring optimal resource allocation in response to hypoxia. Similarly, studies show that NMRs do not suppress kidney metabolism during hypoxia, which coincides with significant oxidative stress responses (255). Specifically, Hadj-Moussa et al. demonstrated that DNA oxidative damage in the kidney significantly increased under both acute (7% $O_2$ for 4 hr) and chronic (7% $O_2$ for 24 hr) hypoxic conditions, and that kidneys were one of the only tissues to activate a robust DNA repair response, including elevated levels of phosphorylated Chk1, ATR, MDM2, and p21 after 4 hr hypoxia. Moreover, kidneys showed increased lipid peroxidation during 24 hr hypoxia exposure, suggesting sustained metabolic activity despite the oxidative burden. These observations underscore that the kidney remains metabolically active under hypoxia and may rely on heightened DNA repair and stress response mechanisms to counteract the accumulation of ROS-induced damage (255).

***Integrating tissue-specific adaptations into whole-animal metabolic strategies.*** At the whole-animal level, these tissue-specific metabolic shifts reflect caste- and age-related physiological priorities, aligning with the functional demands of each developmental stage and social role within

the colony. Subordinates and juveniles actively sustain ATP production through glucose mobilization, enhanced glycolysis, and lactate clearance, supporting higher physical activity levels necessary for burrow maintenance and foraging. In contrast, queens prioritize glucose conservation, limiting carbohydrate utilization and glycolytic flux across tissues, ensuring energy reserves are preserved for reproductive investment rather than immediate energy demands.

These adaptations are likely mediated by hormonal regulation, particularly differences in IGF signaling, reproductive hormones, and metabolic hormones. For example, previous studies indicate that both NMRs and Ansell's mole-rats (*Fukomys anselli*) have significantly lower serum thyroxine (T4) concentrations compared to house mice, a trait that may contribute to their lower basal metabolic rate and body temperature, potentially as an adaptation to subterranean life (256). Typically, mammals exhibit higher serum T4 than T3, but in mole-rats, T4 levels are exceptionally low (257), making this a unique trait among mammals. Similar patterns have been observed in Israeli blind mole-rats (*Spalax ehrenbergi superspecies*) (258) and Merriam's kangaroo rats (*Dipodomys merriami*) (259), both of which also display low plasma free T4 levels compared to mice. This trait is thought to play a key role in regulating metabolic rate and energy expenditure, particularly in response to harsh environmental conditions.

The distinct metabolic response of NMR queens in hypoxia, compared to subordinates and juveniles, may be explained by estrogen-mediated regulation of glucose metabolism and insulin signaling. Queens exhibit higher estrogen levels than subordinates (176), which is known to enhance insulin sensitivity (172, 173). In queens, estrogen enhances insulin signaling efficiency, allowing tissues to maintain glucose homeostasis with fewer insulin receptors and glucose transporters. As a result, queens do not require as many insulin receptors or GLUT transporters

because they respond more effectively to lower insulin levels, contributing to their glucose conservation strategy under hypoxia.

**Future work**. The findings of this study provide significant insights into the glucose metabolism of NMRs under hypoxic conditions. However, there remain several areas for future investigation that could further elucidate the metabolic and physiological adaptations of NMRs.

1. Long-term adaptations to chronic hypoxia

This study primarily examined the effects of acute hypoxia on NMR glucose metabolism. Future research should explore how chronic hypoxia influences long-term metabolic adaptations, particularly regarding insulin and IGF-1 regulation. Investigating metabolic shifts in prolonged hypoxia (e.g., weeks to months) could provide valuable insights into how NMRs maintain homeostasis over extended periods of $O_2$ deprivation.

2. Comparative metabolism across social castes and ages

While this study highlights differences in metabolic responses between subordinate, juveniles and queen NMRs, future studies should investigate how metabolic flexibility varies across different life stages and in male vs. female subordinates. Additionally, understanding how caste-related metabolic differences arise over development could clarify whether metabolic traits are innate or acquired through social and environmental influences.

3. Molecular and genetic regulation of glucose transporters

This study demonstrated changes in the expression of GLUTs under hypoxic conditions. Future studies could examine how gene expression is regulated at the epigenetic level and whether these adaptations are reversible or heritable. Additionally, exploring post-translational modifications of glucose transport proteins may help explain their functional regulation in hypoxia.

4. Investigation of sex hormones during hypoxia

Sex hormones, particularly estrogen, progesterone, and testosterone, play crucial roles in metabolism and physiological adaptation. Hypoxia can influence hormone levels by altering hypothalamic-pituitary-gonadal axis regulation, leading to reproductive and metabolic changes. Future studies should investigate how sex hormones are modulated under hypoxic conditions in NMRs, particularly whether estrogen levels shift to support metabolic flexibility or stress resilience. Understanding the interplay between hypoxia, sex hormones, and metabolism could provide key insights into differential adaptive strategies between sexes and social castes.

5. Metabolomic and proteomic profiling

Using high-throughput metabolomics and proteomics, researchers can identify additional metabolic pathways that may be involved in NMR hypoxia tolerance. Profiling metabolic intermediates across different tissues (brain, liver, muscle, etc.) could uncover novel biochemical adaptations unique to NMRs.

Overall, this study provides novel insights into the metabolic adaptations that may help NMRs thrive under extreme hypoxia, with a specific focus on glucose metabolism across multiple tissues and developmental stages. By examining brain, heart, liver, kidney, and skeletal muscle, we reveal a coordinated yet tissue-specific response to $O_2$ deprivation, emphasizing the interplay between metabolic suppression, substrate prioritization, and energy conservation. A key finding is the divergence between subordinates/juveniles and queens. Subordinates and juveniles enhance glycolysis and glucose uptake to sustain ATP production, supporting their energy-demanding roles in tunneling, foraging, and colony maintenance. In contrast, queens prioritize long-term energy stability through restricted carbohydrate utilization, aligning with their reproductive role and colony stability. These findings position NMRs as a key model for hypoxia adaptation, with

implications for ischemic conditions, stroke, and metabolic disorders in humans. Future studies should explore the genetic and epigenetic regulation of these mechanisms to further understand hypoxia resistance.

**Study limitations.** To further evaluate the reliability of the non-significant findings in chapter 4, post-hoc power analyses were conducted and are summarized in Table 5.1. While several datasets revealed large biological differences, these did not reach statistical significance, likely due to within-group variability and limited sample sizes (n = 4). For example, IGF-2 expression in the juvenile liver had an effect size of 1.39 but would have required n = 8 per group to confidently detect a statistically significant difference. Similarly, datasets such as IGF-2 in the juvenile kidney and IGF-1 in the juvenile kidney were underpowered with n = 4 and would have needed 5–6 replicates to reach adequate statistical power. These findings indicate that although several biologically meaningful trends were observed, such as the 75% reduction in IGF2R brain expression in subordinate and juvenile groups under hypoxia, the small sample size limited the statistical power to detect significance in some cases. If additional replicates had been included in these underpowered datasets, the likelihood of detecting significant differences would have increased. For example, with sufficient sample sizes, we might have confirmed that hypoxia significantly downregulates IGF and insulin receptor expression while upregulating IGF ligands in specific tissues, as indicated in Table 5.1. This pattern suggests a tissue-specific transcriptional response to hypoxia: in the brain, both IGF and receptor expression appear to be consistently downregulated, whereas in peripheral tissues, receptor expression tends to remain unchanged or decrease, while IGF expression is either elevated or stable. Together, these trends support the hypothesis that the brain may suppress energetically demanding processes under hypoxic stress,

potentially to minimize ATP consumption and reduce the generation of ROS, which can accumulate under hypoxia. In addition, sex was not tracked among subordinate animals, which may represent an unmeasured source of variation. It is possible that sex-specific differences, including those influenced by in utero hormone exposure, could affect gene expression and glucose regulatory pathways. Future studies should aim to include sex as a biological variable to clarify whether it contributes to variability within treatment groups.

| Dataset | Effect size | Minimum sample size | Actual power | Interpretation |
|---|---|---|---|---|
| IGF-1 kidney-sub | 2.15 | 4 | 0.85 | Adequate |
| IGF-2 heart-sub | 1.61 | 6 | 0.82 | Marginally sufficient |
| IGF-2 liver-sub | 3.28 | 3 | 0.94 | Adequate |
| IGF1R heart-sub | 2.44 | 4 | 0.92 | Adequate |
| IGF2R brain-sub | 3.56 | 3 | 0.97 | Adequate |
| INSR kidney-sub | 3.54 | 3 | 0.97 | Adequate |
| IGF-1 kidney-juv | 1.64 | 6 | 0.84 | Marginal |
| IGF-2 heart-juv | 1.94 | 5 | 0.87 | Adequate |
| IGF-2 liver-juv | 1.39 | 8 | 0.84 | Minimum acceptable |
| IGF1R heart-juv | 2.41 | 4 | 0.91 | Adequate |
| IGF2R brain-juv | 2.18 | 4 | 0.85 | Adequate |
| INSR kidney-juv | 1.83 | 5 | 0.84 | Marginal |
| IGF-2 heart-queen | 3.07 | 3 | 0.92 | Adequate |
| IGF1R heart-queen | 2.74 | 3 | 0.86 | Adequate |
| INSR kidney-queen | 2.44 | 4 | 0.92 | Adequate |

Table 5.1 Summary of effect size, sample size, and statistical power for selected datasets presented in chapter 4

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