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No Oxygen, No Water: The Unique Physiology
of Annual Killifish Embryos Across Development

by

Daniel Erik Zajic

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Biology

Dissertation Committee:
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Portland State University
2020

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Abstract

For most vertebrates, an abundance of oxygen is necessary for the production of ATP and the maintenance of cellular homeostasis. The absence of oxygen, even for brief periods, quickly leads to ATP depletion which can lead to irreparable damages to sensitive organs, such as the brain and heart. However, certain vertebrates demonstrate an extraordinary ability to thrive and recover fully from periods of no oxygen (anoxia). The annual killifish (*Austrofundulus limnaeus*) lives in ephemeral ponds in the Maracaibo basin of Venezuela and their embryos have the remarkable ability to not only survive anoxic periods for months, but also dehydrating conditions. Survival of this species is dependent on the ability for embryos to enter profound metabolically dormancy termed diapause, as part of their normal life cycle. Survival of dehydrating conditions in *A. limnaeus* embryos is believed to be achieved through reduction of evaporative water loss and thus is likely to highly limit gas exchange. Thus, embryos exposed to dehydration stress may self-impose severe oxygen stress in order to survive without water. There have been many advances in understanding the extreme anoxia tolerance of this system, but little research has been done on dehydration tolerance and how it relates to anoxia tolerance or stress tolerance in general. There is even less known about the evolutionary route that led to increased stress tolerance associated with diapause in *A. limnaeus*. I hypothesize that anoxia tolerance is a pre-adaptation that allowed for the evolution of dehydration tolerance. Thus, I predict that *A. limnaeus* embryos exposed to dehydrating conditions will show similar responses at the molecular level to embryos exposed

to anoxia. A quintessential feature of anoxia-tolerance is the accumulation of neurotransmitter γ -aminobutyric acid (GABA). Despite knowledge of the accumulation of GABA in *A. limnaeus*, little is known about its role in embryos during anoxia or if it plays a role during dehydration stress. The overall goals of this project were to: (1) determine how GABA accumulation relates to stress tolerance of *A. limnaeus* embryos, and (2) explore the physiological mechanisms that allow *A. limnaeus* embryos to survive dehydration stress by monitoring survival, measuring oxygen consumption, and performing a metabolomics analysis.

This project was the first to show that inhibition of GABA production or degradation leads to a reduction in anoxia tolerance. Only embryos that can produce GABA are able to successfully recover from long-term anoxia which may be suggestive of its role as a neurotransmitter, an energy source, and/or an antioxidant during anoxia and aerobic recovery. This project uncovered many new aspects of the biology of *A. limnaeus* embryos during dehydration stress. Diapausing embryos are able to survive extremely dehydrating conditions for over 1.5 years, while developing embryos are able to survive for over 100 days. Embryos in diapause respond to dehydration stress by increasing rates of oxygen consumption while post-diapause II embryos either exhibit the same or reduced rates of oxygen consumption when compared to aqueous embryos. Dormant and actively developing embryos respond to dehydration stress in an active manner by significantly altering their metabolic profile. A number of metabolites accumulate during exposure to dehydration stress that may play an

important role in survival, including the identification of known antioxidants and neuroprotectants. In addition, a number of unique metabolites not yet discussed in the dehydration literature are identified. Despite high oxygen availability, embryos accumulate the anaerobic glycolytic end-product lactate and neurotransmitter GABA. We show that there is undoubtedly overlap in the molecular responses to anoxia and dehydration stress. However, the response to dehydration stress is complex and we have only just begun to scratch the surface into understanding survival of *A. limnaeus* embryos during this crucial time in their life cycle.

Dedication

I dedicate this to anyone that has
ever felt disheartened or not good enough
because of a grade they earned in a class
or by a score they received on a standardized test.

I also want to thank all of the humor and memes
that got me through graduate school, of which I will share
one that embodies my experience in writing this dissertation:

*Writing is that thing at the eye doctor where they say,
“Which of these is better?”
And you can’t actually tell, but you pick one because you have to.
But it’s for every sentence, always, for the rest of your life.*

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The completion of this dissertation would not have been possible without the help and support of so many people. Firstly, I would like to sincerely thank my mentor and advisor Jason Podrabsky for his support and encouragement throughout my project. This dissertation is evidence of what an insightful and caring advisor he is. Jason has always been a positive and reassuring figure for me in and outside of the lab. I thank the members of my committee: Bradley Buckley, Suzanne Estes, Thomas Hancock, and Gwen Shusterman. Their feedback and encouragement in all aspects of research and life has been invaluable. Brad always had words of encouragement and the breaks from science to talk about the Blazers were much appreciated. Suzanne was always very supportive and introduced me to so many amazing undergraduate students that helped me with my work and become a better mentor. Tom showed me what true passion for teaching can lead to and I am forever grateful for everything I have learned from him about physiology. I would like to thank everyone in the biology department at Portland State – students, staff, and faculty – for their support in shaping me into the person I am today.

Much of what molded me into the individual and researcher I am today is attributed to my former labmates, who created a lab environment full of comradery, shenanigans, and baked goods: Amie Romney, Claire Riggs, Cody Steven Woll, and Josiah Wagner. I especially owe a lot to Claire Riggs for her guidance – many days I felt like a lost puppy trying to find his way and she was always there to help. In addition, there have been so many people that have

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Chapter 1

Two fish with one stone?

Model organisms are extensively used in research as they are accessible and convenient systems to study a particular area or question in biology. Traditionally, only a select number of organisms have been widely studied, but with so many advancements in modern technology and research tools, researchers are now able to extend beyond the status quo and explore understudied and unusual organisms. A recent paper by Goldstein and King (2016) argues that some of the biggest future discoveries in biology could come from the development and study of new and atypical model organisms. The origin of model organisms was centralized around wanting to study a particular subset of biology; thus investigating a simple, controllable organism that enables experimental work was logical. Without contributions from studying bacteria, corn, and yeast, much of what we know about basic molecular biology would not exist. A lot of our understanding of developmental biology stemmed from researching flies, worms, *Arabidopsis*, and mice. These systems were mainly chosen as they were smaller, simpler, and faster growing than more complex systems (e.g., humans) (Russell et al., 2017). Goldstein and King (2016) emphasize the need for researchers to ask questions that they know their model organism is appropriate to answer, which was paramount to the development of this dissertation.

For most animals, an ample supply of oxygen and water is essential for survival. Under aerobic conditions, most of the energy (i.e., ATP) used by a cell is captured through oxidative phosphorylation. This process relies on oxygen as the final electron acceptor in an electron transport chain that drives synthesis of ATP through generation of a proton motive force across the inner mitochondrial membrane. Thus, without adequate oxygen, this pathway cannot produce ATP needed for cell survival. Animals respond to a lack of oxygen by shifting energy production to anaerobic mechanisms, typically anaerobic glycolysis. However, glycolysis alone often cannot meet the energetic demands of an organism. A seemingly simple solution to accommodate the low rates of ATP production is to reduce the ATP required by the organism. However, the ability to suppress metabolism and substantially reduce ATP turnover, and thus survive periods of anoxia (no oxygen), in vertebrates is rare. In most organisms, even brief episodes of low oxygen supply can cause irreparable damages to vital organs, such as the brain and heart (Larson et al., 2014). Few vertebrates are able to survive long bouts of no oxygen (anoxia), while even fewer are able to thrive and recover fully from these anoxic periods. In addition, water is essential for life on Earth. Water allows for transportation of materials, function of molecular machinery, and facilitation of chemical reactions. Further, water is important to the structure, stability, dynamics, and function of biological macromolecules. Aquatic organisms have evolved to depend on nearly constant immersion in water to support basic physiological functions. However, in its absence, some aquatic organisms have been able to adapt and survive.

The annual killifish, *Austrofundulus limnaeus*, survives in ephemeral ponds in the coastal deserts of Venezuela (Podrabsky and Hand, 1999). Like most annual killifishes, they have a unique life history that has allowed for the evolution of unique physiological mechanisms in their embryos (See Fig 1.1 for visual representation of *A. limnaeus* life cycle) (Podrabsky et al., 2016). Adult fish are abundant during the rainy seasons, constantly spawning and producing eggs which they deposit into the muddy substrate. While there is an abundance of water, embryos are deposited in the sediment where high microbial activity creates a severely hypoxic or anoxic environment (Podrabsky et al., 1998). Thus, embryos face periods of little to no oxygen availability as part of their normal development. As the ponds dry up and the dry season begins, adult fish die and the embryos must endure the entirety of the dry season until water returns and they can hatch. Embryos now must face dehydrating conditions, the potential for exposure to UV light, extreme temperatures, and salinity stress (Podrabsky et al., 2016). Survival is attributed to the ability of embryos of *A. limnaeus* to enter drastic metabolic dormancy (diapause) as a part of their normal development (Podrabsky and Hand, 1999). Annual killifish embryos can enter diapause at three distinct stages termed diapause I, II, and III. Diapause I (DI) occurs prior to embryonic axis formation at 4 days post fertilization (dpf), diapause II (DII) occurs approximately midway through embryonic development, and diapause III (DIII) occurs at the end of embryonic development prior to attaching (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c). Their anoxia tolerance peaks at DII and is maintained for a few days after breaking diapause II, before decreasing

thereafter (Podrabsky et al., 2007; Podrabsky et al., 2012b; Riggs and Podrabsky, 2017). Thus, *A. limnaeus* provides a gradient of anoxia-tolerant and anoxia-sensitive stages, which allows for a comparative study within a single system.

Respiration during dormancy and rates of water loss are closely related: if oxygen can pass in and carbon dioxide can pass out of the organism, so can water vapor (Danks, 2000). Survival of dehydrating conditions in *A. limnaeus* embryos is achieved through reduction of evaporative water loss and thus is likely to highly limit gas exchange (Podrabsky et al., 2001). Diapausing insects have been seen to prevent water loss by having very low cuticle permeability, which is attributed to the formation of a waxy layer on their cuticle full of long-chain hydrocarbons (Chung and Carroll, 2015; Danks, 2000). To prevent water loss but maintain an adequate amount of oxygen intake and carbon dioxide release, many insects exhibit cyclic respiration. To survive dehydrating conditions, *A. limnaeus* embryos may self-impose severe hypoxia and anoxia in order to maintain their embryonic water content. Despite recent progress on mechanisms of anoxia tolerance in this system, much remains unknown about desiccation tolerance and its relationship with anoxia tolerance or stress tolerance in general. Even less is known about the evolutionary route that led to increased stress tolerance associated with diapause in *A. limnaeus*. *I propose that anoxia tolerance is a pre-adaptation that allowed the evolution of dehydration tolerance. Thus, I predict that A. limnaeus embryos exposed to dehydrating conditions will show similar responses at the molecular level to*

embryos exposed to anoxia. To better understand the potential interplay between the two stresses, we must first look at each stressor individually.

Anoxia

The embryos of the annual killifish *A. limnaeus* exhibit a profound tolerance of anoxia and, when accounting for temperature, they are the most anoxia-tolerant vertebrate known to exist (Fig 1.2) (Podrabsky et al., 2012b). Unlike most developing embryos, *A. limnaeus* displays an increase in anoxia tolerance during development, which peaks at DII (24-32 dpf), is maintained for several days after embryos exit from DII, and then decreases as embryos develop towards hatching. Stress tolerance of *A. limnaeus* embryos in general seems to parallel with their ability to survive anoxia (Machado and Podrabsky, 2007; Podrabsky et al., 2007; Riggs and Podrabsky, 2017; Wagner et al., 2019). The ability to study intraspecific variation in stress tolerance within a single system makes *A. limnaeus* the ideal vertebrate system for comparative stress tolerance studies. The most anoxia tolerant stages (DII and Wourms' stage [WS] 36) are composed mostly of cardiac tissue (functional heart) and neural tissue, which may provide insight into how these tissues, which are the most anoxia-sensitive in other organisms, are able to survive seemingly undamaged after long exposures to anoxia. In addition to comparing anoxia-tolerant and anoxia-sensitive stages within *A. limnaeus*, comparison between the anoxia-tolerant DII and WS 36 stages may shed light on unique mechanisms of survival. The former stage is already metabolically depressed and in arrested development, whereas

the latter is actively developing and must quickly and dramatically reduce their metabolism in response to their environment (enter quiescence) (Podrabsky et al., 2007), thus these embryos may implement separate mechanisms to survive stressful conditions.

Survival during anoxia is attributed to an organism's ability to greatly reduce their metabolic rate. Maintaining adequate ATP production while limiting ATP consumption is paramount for survival in the presence of no oxygen (Hand and Podrabsky, 2000). Metabolic depression is achieved through physiological and biochemical alterations, mainly reductions in protein synthesis, ion regulation, and macromolecule biosynthesis (Fraser et al., 2002; Podrabsky and Hand, 2000; Podrabsky et al., 2007). Protein synthesis accounts for up to 40% of basal metabolism in mammals and up to 90% of total metabolism in fish (Buttgereit and Brand, 1995; Fuery et al., 1998; Podrabsky and Hand, 2000; Smith and Houlihan, 1995). The ability to survive in anoxia is reliant on the embryo's ability to undergo severe metabolic depression, which is largely facilitated by severely depressing protein synthesis. Diapause II embryos maintain high [ATP]/[ADP] ratios and adenylate energy charge during diapause, which supports a coordinated downregulation of ATP production and consumption. This is likely a key to the ability of DII embryos to survive long bouts without oxygen and thus overcome the stress of anoxia. However, when exposed to anoxia, both diapausing and developing embryos experience a drop in ATP levels (by as much as 80%) which is accompanied by an associated drop in heat dissipation (a proxy for metabolism) (Podrabsky et al., 2012a). Thus,

even dormant embryos that are “prepared” for stress must react to anoxia at the metabolic level in order to survive.

In response to anoxia, many anoxia-tolerant organisms produce the neurotransmitter γ -aminobutyric acid (GABA) (Hylland and Nilsson, 1999; Nilsson and Lutz, 1991). GABA has been found to provide excitatory actions in the developing vertebrate nervous system, but conversely, typically functions as an inhibitory neurotransmitter in adults (Ben-Ari, 2002). It has been suggested that GABA (in micromolar concentrations) can act as a mediator of metabolic depression in the anoxic crucian carp brain (Nilsson, 1992). In the anoxia-sensitive mammalian brain, a rapid fall in ATP levels in response to no oxygen leads to general depolarization and release of excitatory amino acids (e.g., glutamate), ultimately concluding in neuronal cell death (Nilsson and Renshaw, 2004). A main factor that differentiates anoxia-tolerant and anoxia-sensitive species is their ability to maintain glutamate homeostasis in response to low oxygen levels. The ability to convert an excitatory neurotransmitter (glutamate) to an inhibitor neurotransmitter (GABA) may be beneficial to organisms trying to depress their metabolism. Anoxia-tolerant vertebrates are able to decrease their metabolic rate by 70% or more during anoxia, while at the same time increasing GABA levels (Nilsson, 1992). Increased GABA levels are thought to protect the brain of many organisms from excitotoxic cell death during hypoxic and anoxic conditions (Podrabsky et al., 2007). However, embryos of *A. limnaeus* accumulate millimolar quantities of GABA during prolonged bouts of anoxia, an amount that is over 1000 times higher than in other anoxia tolerant species

(Podrabsky et al., 2007). It is likely that high glutamate decarboxylase activity can explain the substantial amount of GABA found in embryos exposed to anoxia. The high levels of GABA produced in embryos of *A. limnaeus* suggest that it is likely to act as an inhibitory neurotransmitter at this stage of development and perhaps may serve a purpose other than neurotransmission when embryos are under stress.

Dehydration

Water is essential for life and the threat of dehydration stress puts organisms at risk. However, some organisms have adapted mechanisms for survival when faced with water stress. Many anhydrobiotic organisms accumulate polyhydroxy compounds such as trehalose to essentially “replace” water in order to maintain the hydrogen bonds necessary for structural integrity of biological structures (Crowe and Madin, 1974; Erkut et al., 2011; Wright, 1989). This mechanism allows for the organisms to survive “life without water” and is exhibited in a number of species, including tardigrades (*Hypsibius dujardini*), brine shrimp cysts (*Artemia franciscana*), and the dauer larva stage of *Caenorhabditis elegans* (Crowe et al., 1984; Erkut and Kurzchalia, 2015; Erkut et al., 2011). Alternatively, organisms can reduce water loss behaviorally by burrowing as seen in the African lungfish (*Protopterus aethiopicus*) or by producing a cocoon as seen in some amphibian species (Fishman et al., 1992; Toledo and Jared, 1993).

Embryos of *A. limnaeus* experience unique resistance to desiccation unseen by aquatic vertebrates (Podrabsky et al., 2001). However, dehydration tolerance of these embryos has received much less attention than other aspects of their biology, and many questions remain regarding how long embryos can survive without water and the molecular mechanisms that support survival. Embryos exposed to dehydration stress lose water in the first few days, but by day seven, the rate of water loss is near zero. Importantly, most of this water is lost from the extraembryonic perivitelline compartment, while the embryo appears to remain fully hydrated (Podrabsky et al., 2001). The lack of further water loss suggests embryos may be dramatically reducing gas exchange with their environment to reduce evaporative water loss. As a consequence, reduced permeability to water vapor may lead to highly limited gas exchange in general and thus self-imposed hypoxia or anoxia. This oxygen deprivation may initiate embryonic quiescence (halting development), which is seen in other post-diapause embryos in response to anoxia (Podrabsky et al., 2012b).

Organisms living in aquatic habitats are especially susceptible to low oxygen availability. This is attributed to the low oxygen capacity of water and factors such as salinity, temperature and turbidity affecting oxygen levels. The levels of photosynthesis during the day and night create oxygen fluctuations for aquatic organisms; photosynthetic levels are lower during periods of low light, such as during the night and the winter season, which can create daily hypoxic conditions for aquatic organisms. Thus, hypoxia and anoxia are common elements of many aquatic habits, and many aquatic vertebrates face acute or

chronic periods of low oxygen. *I hypothesize that anoxia tolerance and dehydration tolerance are linked, thus embryos are using the same mechanisms to survive the stress of anoxia as they are to survive desiccation.* Thus, I predict that embryonic stages that have evolved mechanisms to survive low oxygen will have the molecular foundation to survive without water.

Organization of remaining chapters

This work is an exploration of the physiology of *A. limnaeus* embryos in response to two unique stresses: anoxia and dehydration. In Chapter 2, entitled *GABA daba doo, anoxia got nothing on you: GABA metabolism is crucial for long-term survival in annual killifish embryos*, I measure GABA and lactate in *A. limnaeus* embryos across development and during exposure to long-term anoxia and aerobic recovery from anoxia. I use pharmacological inhibitors and transcript data to suggest roles for GABA and lactate during anoxia and recovery. In Chapter 3, entitled *No water, no problem: Stage-specific metabolic responses to dehydration stress in annual killifish embryos*, I assess the dehydration tolerance of embryos across development and then look at rates of oxygen consumption in dormant and actively developing embryos during aerial incubation. I interpret the data as they relate to the ecology of embryonic development in annual killifishes. In Chapter 4, entitled *Metabolomics analysis provides insight on survival of annual killifish (Austrofundulus limnaeus) embryos during dehydration stress*, I perform a metabolomics analysis on embryos exposed to dehydrating conditions to identify potential key metabolites and metabolic pathways that assist in

survival of *A. limnaeus* embryos during dehydration stress. In the final chapter, Chapter 5, entitled *GABA daba done: Summary and future directions*, I review the findings from Chapters 2–4, integrate what I have found as it relates to *A. limnaeus* and organismal stress tolerance, and suggest future directions for research.

Significance

An organism may have a single response to multiple stressors, sharing signaling pathways (cross-talk) or protective mechanisms (cross-tolerance) (Sinclair et al., 2013). Cross-talk occurs when shared regulatory pathways trigger separate mechanisms of protection against different stresses. Conversely, some forms of cellular protection can be effective against multiple forms of stress. A mechanism that can protect against multiple forms of stress is known as cross-tolerance. Understanding the relationship between anoxia and dehydration and the response these stressors induce in *A. limnaeus* will expand the current knowledge of co-evolution and cross-tolerance. I study a system that experiences two major stresses as part of their normal development, which undoubtedly has led to unique physiological adaptations. Together, these chapters aim to unravel the molecular underpinnings behind the physiology of *A. limnaeus* embryos in response to anoxia and dehydration, which will provide insight into how organisms combat different stressors and help explain why *A. limnaeus* thrive where other organisms fail.

Figure 1.1. The life cycle of *Austrofundulus limnaeus*. Adult fish spawn during the rainy season and produce embryos that are able to enter metabolic dormancy (diapause) in three distinct stages as part of normal development. Embryos survive the entirety of the dry season by entrance into diapause II. When the ponds return with the rainy season, embryos continue developing towards hatching.

Figure 1.2. The anoxia tolerance of various vertebrates across development. The embryos of *Austrofundulus limnaeus* (star symbols) exhibit an anoxia tolerance two orders of magnitude higher than any other vertebrate in addition to an initial rise in anoxia tolerance during development. All data has been normalized to 25°C assuming a Q_{10} of 2. The regression analysis performed was calculated without the embryos of *A. limnaeus* and turtle hatchlings, as they were outliers. This figure is from Podrabsky et al. (2012b) and additional details are outlined there.

Chapter 2

GABA daba doo, anoxia got nothing on you: GABA metabolism is crucial for long-term survival in annual killifish embryos

This chapter has previously been submitted for publication:

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Introduction

In most organisms, even brief episodes of low oxygen can cause irreparable damages to vital organs, such as the brain and heart (Larson et al., 2014). Few vertebrates are able to survive long bouts of anoxia, while even fewer are able to thrive and recover from anoxic stress. A small number of vertebrates have evolved the ability to tolerate long-term anoxia, and these organisms share a number of physiological, cellular, and biochemical characteristics that appear to be of fundamental importance for survival without oxygen (Bundgaard et al., 2020; Podrabsky et al., 2007; Riggs et al., 2018; Thompson et al., 2013). One common feature found in all groups of anoxia tolerant vertebrates is the production of GABA, an inhibitory neurotransmitter, in response to anoxia (Hylland and Nilsson, 1999; Nilsson and Lutz, 1991). GABA production in the brain during anoxia is thought to protect against excitotoxic cell death and, generally, relatively small concentrations of GABA (micromolar) are required to

achieve neuroprotection (Gilby et al., 2005; Johns et al., 2000; Lutz and Milton, 2004; Pamenter et al., 2011). Embryos of the annual killifish, *Austrofundulus limnaeus*, can survive for months without oxygen, and long-term survival of anoxia is associated with accumulation of millimolar quantities of GABA (Podrabsky et al., 2007). The accumulation of high concentrations of GABA in anoxic embryos of *A. limnaeus* suggests a role beyond neurotransmission. In this paper we explore the potential role of GABA production in supporting long-term survival of anoxia in *A. limnaeus* embryos.

Austrofundulus limnaeus survives in ephemeral ponds in the coastal deserts of Venezuela by producing embryos that arrest development in diapause as a part of their normal development (Podrabsky and Hand, 1999). Embryos can enter diapause at up to three unique developmental stages, termed diapause I, II, and III, which together extend the time for embryonic development to span the duration of the dry season (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c). Unlike most developing embryos, *A. limnaeus* displays an increase in anoxia tolerance during development, which peaks at diapause II (DII; 24-32 days post fertilization), is maintained for several days after embryos exit from DII, and then decreases as embryos develop towards hatching (Meller et al., 2012; Podrabsky et al., 2007; Riggs and Podrabsky, 2017). At their peak anoxia tolerance, embryos have the remarkable ability to tolerate anoxic conditions for months at 25°C – two orders of magnitude higher than any other vertebrate (Podrabsky et al., 1998; Podrabsky et al., 2012b). What is astounding is that the most anoxia tolerant stages, DII and 4 days post-diapause II (dpd; Wourms'

stage [WS] 36), are composed mostly of characteristically anoxia-sensitive cardiac tissue (functional heart) and neural tissue (Podrabsky and Hand, 1999; Podrabsky et al., 2007). Survival in anoxia is attributed to the ability of embryos to substantially decrease metabolism, either by already being metabolically dormant (in diapause), or through rapidly decreasing metabolism by entering anoxia-induced quiescence (Podrabsky et al., 2012a). Thus, *A. limnaeus* provides a gradient of anoxia-tolerant and anoxia-sensitive stages, which allows for a comparative study within a single system.

Without oxygen, organisms must shift to anaerobic pathways as a means of supporting catabolic metabolism. Most vertebrates rely on anaerobic glycolysis and the production of lactate to fuel metabolism in the absence of oxygen. In most organisms facing anoxia, a lack of adequate glycolytic ATP production to meet metabolic demand leads to a bioenergetic and redox imbalance and the activation of cell-death pathways (Boutillier, 2001; Jacobson and Raff, 1995). Even if metabolic supply and demand are balanced, survival times in anoxia are typically limited by the negative effects of lactic acidosis, which can be partially offset through increased buffering capacity (Jackson, 1997; Jackson et al., 2000). The ability for *A. limnaeus* embryos to maintain high [ATP]/[ADP] ratios during diapause supports a coordinated downregulation of ATP production and consumption, and is likely a key to the embryos ability to survive long bouts without oxygen during diapause. However, ATP levels are not maintained in developing embryos exposed to anoxia (Podrabsky et al., 2012a), and yet these embryos exhibit similar survival of anoxia (Podrabsky et al., 2007). Thus, in *A.*

limnaeus there may be multiple mechanisms for achieving extreme anoxia tolerance. Recent progress has been made on understanding multiple factors that may be contributing to the survival of *A. limnaeus* in anoxia (depressed protein synthesis, inhibition of apoptosis, antioxidant capacity, and small noncoding RNAs) (Meller and Podrabsky, 2013; Podrabsky and Hand, 2000; Riggs and Podrabsky, 2017; Wagner et al., 2019).

When exposed to anoxia, embryos of *A. limnaeus* respond by producing large amounts ($> 10 \text{ mmol l}^{-1}$) of GABA (Podrabsky et al., 2007). GABA is the primary inhibitory neurotransmitter in the adult central nervous system; however, it appears to function in an excitatory manner during early vertebrate development (Ben-Ari, 2002). The high levels of GABA produced in anoxic embryos of *A. limnaeus* suggest a role beyond inhibitory neurotransmission. Despite the massive accumulation of GABA in *A. limnaeus* embryos, the metabolic origins and function of GABA in *A. limnaeus* embryos remain unexplored.

Here we report on the long-term metabolic responses of dormant (DII) and actively developing post-diapause II embryos to anoxia and aerobic recovery to evaluate the role of GABA. We evaluate these data in relation to anoxia tolerance by utilizing developmental stages that provide a gradient of anoxia tolerance. We inhibit enzymes responsible for GABA production and degradation to see how GABA and lactate levels are affected and how that affects survival in anoxia. These data support a role for GABA as a metabolic intermediate, and not a metabolic end-product. Here we suggest multiple roles for GABA during anoxia

and aerobic recovery in *A. limnaeus* embryos: as a neurotransmitter, as a source of energy, and as an antioxidant. We support our findings with transcript expression data during normal *A. limnaeus* embryonic development and in response to anoxia and aerobic recovery. We explore how long-term survival in anoxia and subsequent aerobic recovery may be supported by accumulation of GABA. Understanding how the most anoxia tolerant stages, composed mostly of cardiac tissue and neural tissue, utilize GABA may provide insight into how these tissues are able to survive long-term anoxia.

Materials and Methods

Husbandry of A. limnaeus and embryo collection

Adult annual killifish (*Austrofundulus limnaeus*) were reared in the Portland State University (PSU) aquatic vertebrate facility and cared for according to established husbandry methods in accordance with approved PSU Institutional Animal Care and Use Committee protocols (PSU IACUC protocols #33 and 64). Adult *A. limnaeus* were kept in spawning pairs and housed in 9.5 l tanks and collectively 21 tanks were connected to a communal sump and filtration system. Paired adults were allowed to spawn semiweekly and embryos were collected. Embryos were stored at 25°C under dark conditions in 15 x 100 mm plastic Petri dishes in media that resembles the environmental conditions from which adults were collected in 1995 (10 mmol l⁻¹ NaCl, 2.15 mmol l⁻¹ MgCl₂, 0.8 mmol l⁻¹ CaCl₂, 0.14 mmol l⁻¹ KCl, 1.3 mmol l⁻¹ MgSO₄) (Podrabsky, 1999; Podrabsky et al., 1998). Embryo medium contained methylene blue (0.0001%)

for the first 4 days post-fertilization (dpf) to prevent fungal growth. To prevent microbial growth and infection, embryos were treated at 4 dpf with two 5 min rinses of embryo medium containing sodium hypochlorite (Clorox, 0.03%) separated by a 5 min rest in embryo medium without sodium hypochlorite. After the last sodium hypochlorite wash, embryos were rinsed with embryo medium containing sodium thiosulfate (0.005%) to neutralize remaining sodium hypochlorite. Embryos were then placed in medium containing 10 mg l⁻¹ gentamicin sulfate and allowed to develop at 25°C without light to promote entry into DII and prevent breaking of DII until experiments were conducted (28–66 d) (Podrabsky et al., 2010a; Romney et al., 2018). Embryos enter DII at around 24 dpf at 25°C.

Developmental staging and sampling of embryos

Diapause II was broken by subjecting embryos to a temperature of 30°C and full spectrum light for 48 h (Meller et al., 2012). Upon breaking DII, embryos were sorted by developmental stage according to Wourms' stage (WS) in concert with recent adaptations by Podrabsky et al. (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c). Experiments were performed on four stages of embryos to capture a gradient of anoxia tolerance levels and physiology: DII (Diapause II, WS 32/33), WS 36 (4 days post-diapause II (dpd)), WS 40 (12 dpd), and WS 42 (20 dpd). DII embryos are metabolically dormant and have arrested development and thus are primed for anoxia; whereas, post-DII embryos are metabolically and developmentally active and enter quiescence in response to anoxia (Podrabsky

et al., 2007). Lethal time to 50% mortality (LT_{50}) in response to anoxia has previously been described (Meller et al., 2012; Podrabsky et al., 2007; Riggs and Podrabsky, 2017). Embryos develop anoxia tolerance as they enter DII (LT_{50} = 65 d) and maintain extreme tolerance for 4–6 days after breaking DII, after which anoxia tolerance decreases dramatically in later stage embryos: WS 36 (LT_{50} = 74 d), WS 40 (LT_{50} = 7 d), WS 42 (LT_{50} = 0.67 d). Embryos were sampled in their respective environmental conditions (anoxia or normoxia) by placing them on a mesh screen and wicking away excess liquid using dry paper towels prior to being placed in a pre-weighed microcentrifuge tube. Samples were then quickly weighed and stored at -80°C until metabolite extraction. Typically, embryos were used from a single spawning event to represent a single replicate; however, in some instances multiple spawning events were combined in order to have sufficient embryos at a specific developmental stage ($N = 3-6$). Sampling of embryos covered the lifespan of *A. limnaeus* and multiple generations, however, control embryos were always sampled to account for any batch or generational effects.

Long-term anoxia exposure, recovery, and sampling

Embryos were exposed to various periods of anoxia based on their anoxia tolerance, such that the longest period of anoxia matched their LT_{50} (Fig 2.1A). Embryos were allowed to recover following long-term anoxia and samples were collected at several time points following aerobic recovery. WS 42 embryos either hatched or were not able to recover following reoxygenation and data for

recovery were not collected. Embryos were exposed to anoxia in a Bactron III anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA), which maintained anoxic conditions by using hydrogen gas and a palladium catalyst to react with any trace oxygen. Anoxic conditions were maintained at 5% CO₂, 5% H₂, and 90% N₂ with temperature held constant at 25°C with no light. Embryos were first removed from their normoxic medium, transferred into the chamber through an air lock, and placed in anoxic medium that had been previously bubbled with N₂ gas for 30 min and equilibrated overnight to conditions in the anoxic chamber. Samples were collected as biological replicates consisting of five embryos each ($N = 4$ for DII, WS 36, and WS 40; $N = 3$ for WS 42).

Pharmacological inhibition of GABA synthesis and degradation

Three inhibitors of canonical GABA synthesis and degradation were used (Fig 2.2A). Ethyl ketopentenoate (EKP) is a lipid-permeable and specific inhibitor of glutamate decarboxylase (GAD) (Zhang et al., 2017). Inhibition of GAD occurs through binding of EKP to the native glutamate active site of both isoforms of GAD (GAD67 and GAD65). EKP was synthesized and gifted to us from Dr. Peter A.M. de Witte and his lab (KU Leuven, Leuven, Belgium). Vigabatrin (vinyl- γ -aminobutyric acid) (V8261, Sigma Aldrich, St. Louis, MO, USA) is a selective, irreversible inhibitor of GABA transaminase (GABA-T; ABAT), the major enzyme that synthesizes the first step in the metabolic degradation of GABA (Ben-Menachem, 2011; Qume and Fowler, 1997; Sloley et al., 1994; Wu et al., 2001). N,N-diethylaminobenzaldehyde (DEAB) (D86256, Sigma Aldrich, St. Louis, MO,

USA) is a selective inhibitor of aldehyde dehydrogenase isoenzymes (Koppaka et al., 2012; Morgan et al., 2015). Succinic acid semialdehyde dehydrogenase (SSADH) is part of the aldehyde dehydrogenase 5 family (ALDH5A1), a target of DEAB (Morgan et al., 2015). Embryos of *A. limnaeus* are generally highly impermeable to external substances. Consequently, higher concentrations of inhibitors and the addition of dimethyl sulfoxide (DMSO) in the embryo medium are used to achieve biologically active concentrations within embryos (Podrabsky and Hand, 2000; Pri-Tal et al., 2011; Woll and Podrabsky, 2017). Stocks of pharmacological inhibitors were prepared in DMSO and diluted in embryo medium without antibiotics, yielding a final concentration of 1% DMSO. The final concentrations of inhibitors used in the experiments were: 400 and 800 $\mu\text{mol l}^{-1}$ EKP; 1 mmol l^{-1} Vigabatrin; 167 $\mu\text{mol l}^{-1}$ DEAB. Preliminary range-finding experiments were used to determine these concentrations in addition to observed concentrations used in the literature. The potential toxicity of each of the inhibitors was tested on WS 36 embryos. Embryos were treated with each of the inhibitors in normoxia and monitored for at least 7 d with daily media changes to observe any developmental defects or mortality. For anoxic exposures, groups of 15 WS 36 embryos ($N = 3$) were placed in clear 12-well polystyrene plates (CytoOne, CC7682-7512, USA Scientific, Ocala, FL, USA) and pre-exposed to embryo medium containing each inhibitor for 2 h at 25°C without light. Embryos were then transferred into the anoxic chamber where fresh anoxic embryo medium containing each inhibitor was replenished. Concentrated stock solutions (EKP, Vigabatrin: 100 mmol l^{-1} ; DEAB: 25 mmol l^{-1}) of each inhibitor were

prepared under anoxic conditions and stored at -80°C . These stocks were diluted daily within the anoxic chamber to make fresh embryo medium, which was changed daily. Embryos were monitored daily for survival until 100% mortality was reached. A separate plate of embryos was used for metabolite sampling purposes and embryos were sampled at 0, 7, 14, and 30 d ($N = 3$). Each replicate contained five embryos. To determine if a non-canonical pathway through polyamine metabolism was contributing to GABA production during anoxia, embryos were treated with amiloride. Amiloride is an inhibitor of diamine oxidase, the enzyme that catalyzes the penultimate step in GABA production by converting putrescine to 2-aminobutanal (Fig 2.2A). Amiloride ($100\text{ }\mu\text{mol l}^{-1}$) treated embryos were only sampled at 0 and 8 d ($N = 4$).

Metabolite extraction

Metabolites were extracted from whole embryos following previously detailed protocols (Podrabsky and Hand, 1999) and briefly described here. Samples were removed from -80°C and immediately homogenized (1:7 w/v) in ice-cold 1 mol l^{-1} perchloric acid containing 5 mmol l^{-1} EDTA. Samples were then subjected to centrifugation at $16,000\text{ g}$ for 15 min at 4°C to pellet macromolecules and other insoluble cellular components. Supernatants were removed and neutralized with 9% volume of ice-cold 5 mol l^{-1} K_2CO_3 and perchlorate precipitates were removed by centrifugation at $16,000\text{ g}$ for 30 min at 4°C . Supernatants were then transferred to new tubes and frozen at -80°C until GABA and lactate determinations were performed.

γ -aminobutyric acid (GABA) determination

GABA levels were enzymatically measured using a spectrophotometric assay that was modified from previously established protocols using a 96 well plate format (Ippolito and Piwnica-Worms, 2014; Tsukatani et al., 2005). Samples (10 μ l) were added to wells of a black clear bottom 96 well plate (Corning™ 3603, 07-200-565, Fisher Scientific, Hampton, NH, USA), followed by addition of 90 μ l of a freshly prepared reaction mix containing: 0.1 mol l⁻¹ Tris-HCl (pH 8.8), 10 mmol l⁻¹ DTT, 1.4 mmol l⁻¹ NADP, 2.0 mmol l⁻¹ α -ketoglutarate, and 0.126 U ml⁻¹ GABase (G7509, Sigma Aldrich, St. Louis, MO, USA). Change in absorbance was recorded every 60 s for 90 min at 340 nm (A_{340}) to monitor NADPH production (Tecan Infinity M200Pro, Männendorf, Switzerland). Because any endogenous SSAL found in the sample would give false GABA levels, an inhibitor of GABA-T, 2-aminoethyl hydrogen sulfate (2-AEHS) (TCS0445, VWR, Radnor, PA, USA), was used to account for any signal resulting from endogenous SSAL being converted to GABA in the assay. An inhibitor reaction mix was created containing 50 mmol l⁻¹ 2-AEHS. The reaction mix was allowed to incubate for 10 min following addition of GABase but before addition of the sample to ensure sufficient time for GABA-T inhibition. The final concentration of GABA was determined by subtracting the amount of GABA measured in the presence of inhibitor from the total quantity of GABA detected in the assay. Both GABA and SSAL standards were used to calculate concentrations using a standard curve. A standard curve was generated for each assay and A_{340} increased in a linear fashion with increasing GABA concentrations ($R^2 = 0.99 \pm$

0.001, $N = 41$ assays). SSAL concentrations were generally low in the samples and never represented more than 5% of the total GABA measured.

Lactate determination

Lactate levels were enzymatically measured following a previously detailed protocol, but modified for use in a 96 well plate format (Gleeson, 1985). Each well contained the following: 218 μl of Glycine(0.6 mol l^{-1})-Hydrazine(0.5 mol l^{-1}) buffer (pH = 9.2), 25 μl 27 mmol l^{-1} NAD^+ , 5 μl of lactate standard or sample, and 2.5 μl 14 U ml^{-1} lactate dehydrogenase (LDH; L2625, Sigma Aldrich, St. Louis, MO, USA). The addition of LDH catalyzed the oxidation of lactate to pyruvate and the associated production of NADH was monitored spectrophotometrically at A_{340} for 60 min (Tecan Infinity M200Pro). The final concentration of lactate was determined by interpolating sample A_{340} to the lactate standard curve ($R^2 = 0.99 \pm 0.0004$, $N = 25$ assays).

Glutamate decarboxylase (GAD) activity

Glutamate decarboxylase (GAD) activity was measured following a previously detailed protocol with modifications (Wolf and Klemisch, 1991). Whole embryos in groups of 13 ($N = 4$) from 4 developmental stages (DII, WS 36, WS 40, and WS 42) were removed from -80°C and immediately mechanically homogenized with a Teflon pestle (1:3 w/v) in ice-cold 10 mmol l^{-1} potassium phosphate buffer (pH 7.0) containing 1 mmol l^{-1} 2-(2-aminoethyl)isothiourea dihydrobromide (AET), 0.1% Triton X-100, and 250 $\mu\text{mol l}^{-1}$ pyridoxal 5'-

phosphate (PLP). Samples were then sonicated on ice at 25% amplitude for 15 s to disrupt cells (Branson Digital Sonifier, S-450D, 1/8" SF 150 microtip probe). Samples were then subjected to centrifugation at 5,000 **g** for 30 min at 4°C. Supernatants containing GAD were removed and frozen at –80°C until the enzyme assay could be run. Preliminary experiments showed no difference in GAD activity between fresh and previously frozen samples. Samples were removed from –80°C and allowed to thaw at room temperature for 5 min and then placed on ice for 10 min. Samples were then subjected to centrifugation at 5,000 **g** for 30 min at 4°C prior to measuring activity. Supernatants were removed and 10 µl were mixed with 10 µl of GAD-substrate solution (0.1 mol l⁻¹ potassium phosphate buffer, pH 8.0, containing 50 mmol l⁻¹ glutamate, 250 µmol l⁻¹ PLP, and 0.4% 2-mercaptoethanol). Samples were incubated at 25°C without light for 0, 6, 12, and 24 h. Samples (*N* = 4) containing 10 µl water and 10 µl GAD-substrate solution were used as negative controls. Reactions were terminated by addition of 10 µl 0.25 mol l⁻¹ HCl and then placed on ice for 5 min. Samples were then frozen at –80°C until GABA concentrations were determined as described above. To determine the efficacy of our assay in measuring GAD activity, we measured activity in adult *A. limnaeus* brains that were dissected from 2 males and pooled together. Brain samples (*N* = 1) were subjected to the same sample preparation as embryos. Following the addition of GAD-substrate solutions (pH 6.0, 7.0, 8.0, or 9.0), samples were incubated at 25°C without light for 0, 0.75, 1.5, and 3 h and GABA accumulation was measured as described above.

RNAseq data and identification

Gene transcripts associated with GABA production, signaling, degradation, transport, and binding, as well as polyamine metabolism, lactate production and transport, and ethanol production were identified from the *A. limnaeus* genome annotation version 100 in the NCBI GenBank database (Wagner et al., 2018). Transcript expression data were obtained from datasets deposited in GenBank (Bioproject PRJNA272154). Data for early embryos developing at 20°C that will enter into DII ($N = 3$) are from Romney et al. (2018). Data for DII and post-DII embryos are from embryos incubated at 25°C and exposed to short-term anoxia. The data were collected as outlined previously (Riggs and Podrabsky, 2017). Briefly, DII, WS 36, and WS 40 embryos were sampled after 4 and 24 h of anoxia and after 2 and 24 h of aerobic recovery ($N = 4$ for DII and WS 36; $N = 6$ for WS 40). WS 42 embryos were sampled at 2 and 6 h of anoxia followed by 2 and 24 h of aerobic recovery ($N = 4$). RNA sequencing libraries were constructed using the Illumina TruSeq RNA Sample Preparation Kit (v2, Illumina, San Diego, CA, USA) following the manufacturer's instructions. Libraries were sequenced (100 bp paired-end reads) on a HiSeq 2000 (Illumina) instrument as outlined in Romney and Podrabsky (2017). Some of these data have been previously published (Wagner et al., 2018).

Statistical analysis

Graphical and statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA, USA) and SPSS software (IBM., v.26.0,

Armonk, NY, USA). LT₅₀ of embryos exposed to inhibitors in anoxia were calculated by probit regression analysis (Chapman et al., 1995) using SPSS software. Where appropriate, analysis of variance (ANOVA), analysis of covariance (ANCOVA), unpaired t-test, correlation analysis, linear regression, segmental linear regression, or exponential one-phase decay nonlinear regression were used. Nonlinear and segmental linear regressions reported only include coefficient of determination (R^2) values, as probability (P) values cannot be calculated. To support whether slopes calculated are significantly non-zero, linear regressions were initially performed and were significant ($P < 0.05$). Tukey's honestly significant difference (HSD) test and Dunnett's test were used for *post-hoc* comparisons, where applicable. Statistical significance was set to $P < 0.05$ for all comparisons.

Results

Long-term anoxia exposure and recovery

Lactate

All developmental stages of *A. limnaeus* responded to anoxia by accumulating significant amounts of lactate (Fig 2.1B; one-way ANOVA, $P < 0.0001$). To account for clear differences in initial and final rates of production, segmental linear regressions were performed for each developmental stage (Fig 2.1B; Table 2.1). Initial rates were higher than final rates for each stage of embryo (Table 2.1). Rates were lowest in DII and increased with further post-DII development (one-way ANOVA with Tukey's HSD, $P < 0.01$). WS 36 embryos

accumulated the highest amount of lactate during anoxia (Fig 2.1B; 23.6 ± 1.09 mmol kg⁻¹ embryo; mean $\pm$ SEM, $N = 4$), followed by WS 40 embryos (18.7 ± 3.07 mmol kg⁻¹ embryo). However, WS 40 embryos accumulated lactate at a rate seven times faster than WS 36 embryos (Table 2.1). We observed a significant correlation between initial rates of lactate accumulation and LT₅₀ in anoxia (Fig 2.2A). The relationship between initial rates of lactate accumulation and LT₅₀ in anoxia reported here on DII and post-DII embryos is similar to those from previous experiments on pre-DII and DII embryos (Fig 2.2A; ANCOVA, $P = 0.1886$) (Podrabsky et al., 2007). For those embryos that recover from anoxia, lactate slowly returned back to initial levels after several days to a week (DII, WS 36 and WS 40; Dunnett's *post-hoc*, $P < 0.05$).

GABA

There was significant accumulation of GABA in DII, WS 36, and WS 40 embryos, while WS 42 embryos showed no significant accumulation (Fig 2.1B; one-way ANOVA, $P < 0.0001$ for DII, WS 36 and WS 40; $P = 0.2286$ for WS 42). There were clear initial and final rates of GABA accumulation in DII, WS 36, and WS 40 embryos, so segmental linear regressions were performed (Fig 2.1B; Table 2.1). Regression analysis of data for WS 42 embryos confirmed a slope that was not statistically different from zero (Table 2.1). Initial rates of GABA accumulation were higher than final rates in DII and WS 36 embryos (Table 2.1). In contrast, final rates were higher than initial rates in WS 40 embryos (unpaired t-test, $P < 0.05$). Rate of GABA accumulation was lowest in DII embryos and

increased with post-DII development in WS 36 and WS 40 embryos. There was no relationship found between rates of initial or final GABA accumulation and LT_{50} in anoxia (Fig 2.2B; correlation analysis, initial: $r = 0.10$, $P = 0.8951$; final: $r = -0.34$, $P = 0.6561$). An inverse relationship was found in the ratio of lactate to GABA accumulated at the LT_{50} when compared to the LT_{50} of each stage (Fig 2.2C). During aerobic recovery from anoxia, GABA slowly returned to initial levels in DII and WS 40 embryos (Dunnett's *post-hoc*, $P < 0.05$).

Toxicity of pharmacological inhibitors in normoxia

In normoxia, WS 36 embryos incubated in GAD inhibitor EKP ($400 \mu\text{mol l}^{-1}$), GABA-T inhibitor vigabatrin, SSADH inhibitor DEAB, and DAO inhibitor amiloride survived, developed normally, and had developmental rates that were indistinguishable from those of control embryos (data not shown). In contrast, embryos exposed to $800 \mu\text{mol l}^{-1}$ EKP showed mortality following 5 d of exposure (Fig 2.3). However, it should be noted that embryos continue developing in normoxia, which is not the case during exposure to anoxia. Additionally, embryos exposed to $800 \mu\text{mol l}^{-1}$ EKP survived in anoxia at WS 36 significantly longer than developing embryos exposed to $800 \mu\text{mol l}^{-1}$ EKP in normoxia (Fig 2.5B,C; Fig 2.3). Exposure to $800 \mu\text{mol l}^{-1}$ EKP starting at WS 40 led to mortality after 2 d (Fig 2.3). These data are interpreted to indicate stage-specific toxicity of EKP in WS 39 and later embryos and likely illustrate a necessity for GABA in late development.

Inhibition of GABA synthesis and degradation in anoxia

Ethyl ketopentenoate (EKP)

Ethyl ketopentenoate, a potent inhibitor of GAD, exhibited dosage-dependent reductions in GABA production during anoxia in WS 36 embryos (Fig 2.4B,C). GABA levels were significantly lower after 14 d in 400 $\mu\text{mol l}^{-1}$ treated embryos when compared to controls, while levels were significantly lower after only 7 d in 800 $\mu\text{mol l}^{-1}$ treatments (Fig 2.4B,C; two-way ANOVA with Tukey's HSD, $P < 0.05$). Embryos exposed to 400 $\mu\text{mol l}^{-1}$ EKP produced GABA at a rate 42% slower than control embryos; whereas, embryos exposed to 800 $\mu\text{mol l}^{-1}$ EKP produced GABA at a rate 52% slower than controls. Interestingly, exposure to either concentration of EKP led to no changes in rates of lactate accumulation (Fig 2.4D,E; two-way ANOVA with Tukey's HSD, $P > 0.05$; ANCOVA, $P > 0.05$). However, exposure to both dosages of EKP led to decreased survival in anoxia (Fig 2.5A,B). When the survival data were used to calculate LT_{50} values, embryos exhibited a 37% and 44% reduction in survival compared to controls, with LT_{50} values of 44 ± 4 and 39 ± 2 d (mean $\pm$ SEM, $N = 3$) in anoxia for 400 and 800 $\mu\text{mol l}^{-1}$ treatments, respectively (Fig 2.5C). Despite having similar mean LT_{50} values (one-way ANOVA, $P = 0.78$), 800 $\mu\text{mol l}^{-1}$ treated embryos did not survive as long as 400 $\mu\text{mol l}^{-1}$ treated embryos (Fig 2.5B,C). Embryos treated with 400 $\mu\text{mol l}^{-1}$ reached 100% mortality after 62 d, while it only took 51 d for 800 $\mu\text{mol l}^{-1}$ treated embryos.

Vigabatrin

Vigabatrin, an inhibitor of GABA-T, increased GABA levels in WS 36 embryos exposed to anoxia after 14 d compared to control embryos (Fig 2.4C; two-way ANOVA with Tukey's HSD, $P < 0.05$). GABA accumulated at a rate 23% faster than controls. Lactate accumulation was not affected by vigabatrin (Fig 2.4E; two-way ANOVA with Tukey's HSD, $P > 0.05$; ANCOVA, $P > 0.05$). Vigabatrin decreased survival of anoxic embryos by 20% ($LT_{50} = 56 \pm 0.5$ d; mean $\pm$ SEM, $N = 3$; Fig 2.5B,C).

N,N-diethylaminobenzaldehyde (DEAB)

DEAB, an inhibitor of SSADH, increased GABA levels in WS 36 embryos exposed to anoxia after 30 d compared to control embryos (Fig 2.4B, two-way ANOVA with Tukey's HSD, $P < 0.05$). GABA accumulated at a rate 26% faster than controls. DEAB had no effect on rates of lactate accumulation (Fig 2.4D; two-way ANOVA with Tukey's HSD, $P > 0.05$; ANCOVA, $P > 0.05$). DEAB decreased survival of anoxic embryos (Fig 2.5B), leading to a 44% reduction in LT_{50} (39 ± 4 d; mean $\pm$ SEM, $N = 3$; Fig 2.5C).

Amiloride

Amiloride, an inhibitor of DAO, did not affect GABA levels in WS 36 embryos exposed to anoxia for 8 d (Fig 2.6).

Glutamate decarboxylase (GAD) activity during development

Significant rates of GABA synthesis were observed in homogenates of adult brain tissue incubated at pH 7 – 9, confirming the utility of this assay for estimating GAD activity in homogenates of *A. limnaeus* (Fig 2.7; regression analysis, $P < 0.005$). We observed low, but significantly greater than zero, rates of GAD activity in post-DII embryo homogenates (Fig 2.8A). No significant GAD activity was detected in DII embryos after 24 h of incubation (Fig 2.8A). Despite the low observed GAD activity, this capacity for GABA production is sufficient to account for the GABA produced in whole embryos during anoxia. Observed GAD activity provides reasonable prediction of initial GABA accumulation during anoxia ($LT_{50}/4$) for DII and WS 36 embryos (Fig 2.8B; unpaired t-test, $P > 0.05$). In contrast, the capacity for GABA production by GAD is significantly greater than needed in WS 40 and WS 42 embryos (unpaired t-test, $P < 0.05$). Despite observing the highest enzymatic capacity for GAD activity, WS 42 embryos did not produce significant levels of GABA during anoxia (Fig 2.1B). There is a significant negative relationship between LT_{50} and GAD activity (Fig 2.8C).

RNAseq

GABA pathway

Early developing embryos had generally higher levels of transcripts involved in GABA synthesis and degradation, which tended to decrease and level off as they approached DII (Fig 2.9A). DII embryos observed relatively constant expression of these transcripts during anoxia and recovery. Interestingly GAD2

expression was highest in DII, whereas it was absent or low in expression in all other stages. WS 36 embryos exhibited highest expression of GAD1 in post-DII embryos, which increased with short term anoxia exposure and decreased with aerobic recovery. Transcripts for GABA-T decreased with anoxia and recovery, but were still expressed (> 1000 FPKM). SSADH transcripts levels were maintained during anoxia and recovery in DII and post-DII stages. WS 40 embryos have relatively high GAD1 and GABA-T expression. The overall expression pattern for GABA synthesis and degradation in WS 42 embryos was relatively high and constant during anoxia, despite not producing GABA. However, expression of GAD transcripts increased during aerobic recovery. GAD enzyme activity (Fig 2.8C) is inversely related to transcript expression patterns for GAD enzymes in post-DII embryos.

Polyamine metabolism

A number of transcripts involved in polyamine metabolism were abundant in all embryonic stages (Fig 2.9B). The most abundant transcripts (*SAT1*, *ODC1*, and *ALDH9A1*) remained high throughout development. A transcript that encodes a diamine oxidase (*AOC2*) was highest in WS 20 embryos and decreased with development towards DII, where it then increased in abundance and remained high during DII and post-DII development.

Lactate and ethanol pathways

Transcripts for lactate dehydrogenases (LDH) were abundant in all stages of embryos (Fig 2.9C). LDH B was the primary isoform expressed in early embryos, while both the A and B isoforms were expressed in DII. For post-DII embryos, LDH B was the primary transcript expressed with LDH A appearing in WS 36 embryos during recovery from anoxia. Transcripts for proteins involved in ethanol production were low in DII and post-DII embryos, with the most abundant transcripts (Fig 2.9C; *PDHB*, *PDK2A*, *DLD*, and *ADH3*) showing similar expression patterns in all stages, with the exception of an *ADH1*-like transcript being highly expressed in WS 40 embryos.

GABA and lactate transporters

GABA transporters (GAT) were absent or expressed in low levels during early development (Fig 2.9D). In contrast, transcripts for GAT1 and GAT3 paralogs were highly expressed in DII embryos. These same transcripts were induced during anoxia and aerobic recovery in WS 36 embryos. WS 40 embryos uniquely only expressed GAT2 paralogs, whereas WS 42 embryos expression patterns were similar to DII embryos, but at lower levels. An abundance of monocarboxylate transporters (MCTs) were expressed in embryos of *A. limnaeus* (Fig 2.9D). Early developing embryos displayed the highest expression of MCTs, primarily expressing MCT1-like and MCT7-like transcripts, which were highest in WS 20 embryos and decreased with development towards DII. Generally, DII embryos exhibited more MCT expression than post-DII embryos.

GABA receptors and cotransporters

The *A. limnaeus* genome contains an abundance of GABA_A receptor subunit variants compared to relatively few GABA_B receptor subunit variants (Fig 2.10). Despite the high expression of transcripts involved in GABA synthesis and degradation, transcripts for GABA signaling were predominantly absent in early developing embryos, with the exception of a single GABA_AR subunit- ρ -3-like transcript. In contrast, DII embryos expressed the highest diversity of GABA receptor subunits. WS 36 embryos had relatively low amounts of expression prior to anoxia, which consistently increased during anoxia and aerobic recovery. WS 40 embryos had similar expression to early developing embryos, with most receptors being minimally expressed besides GABA_AR subunit- ρ -3-like. WS 42 embryos had consistent expression of several receptors that generally matched the receptors expressed in DII embryos, some of which increased during recovery from anoxia. GABA_A receptor-associated proteins were highly expressed in all stages (Fig 2.10). The highest expression of GABA_A receptor-associated proteins was found in WS 20 embryos. Cotransporters (NKCC and KCC) were observed in all stages of embryos (Fig 2.10). KCC1 expression was low early in development, but increased as embryos approached DII and remained constant during post-DII development. Interestingly, NKCC2 was highly expressed in DII embryos, but barely expressed in all other stages.

Age of female on GABA and lactate accumulation

Sampling of embryos spanned the lifespan of *A. limnaeus* and across generations. Due to differences in GABA and lactate levels observed between experiments, we explored the relationship between female age and rates of GABA and lactate accumulation in anoxic WS 36 embryos. No correlation was found between the rate of GABA accumulation and female age (Fig 2.11A; $R^2 = 0.07$, $P = 0.67$). Interestingly, embryos from younger females produced lactate during anoxia at a faster rate than embryos produced by older females (Fig 2.11B; $R^2 = 0.97$, $P < 0.01$).

Discussion

The impressive survival of anoxia in *A. limnaeus* embryos appears to rely fundamentally on the same metabolic pathways as anoxia-sensitive organisms with lactate as the major product of anaerobic glycolysis. Consistent with previous data for early embryos of *A. limnaeus* (Podrabsky et al., 2007) and a variety of other organisms (Hand, 1998), survival of anoxia in DII and post-DII embryos is proportional to the level of metabolic depression achieved. This point is illustrated well by the high negative correlation between rate of lactate accumulation and survival of anoxia as estimated by the LT_{50} . Thus, the fundamental key to long-term survival of anoxia appears to be the limiting of lactate accumulation.

Lactic acidosis is a major factor in contributing to cell death in low oxygen environments (Kubasiak et al., 2002). Fish embryos are a closed system, which

is especially important because metabolic end-products, such as lactate, have no means of exiting the embryo. In the western painted turtle, lactic acidosis is buffered by the turtle's mineralized shell (Jackson, 1997; Jackson et al., 2000; Jackson and Heisler, 1982). The internal pH of *A. limnaeus* embryos has never been measured. Although the embryos have a high tolerance of a wide range of external pH conditions and can survive for about a month at pH 2 (Podrabsky et al., 2016), it is unclear whether embryos have high capacity to buffer the acidosis of lactate or if they are able to tolerate a low internal pH. The robust expression of several lactate transporters suggests lactate is likely being shuttled within the embryo. A likely destination for lactate is the yolk. Shuttling lactate into the yolk may prevent embryonic cells from experiencing severe acidosis. However, additional work is needed to explore the buffering capacity of the yolk and possible compartmentalization of metabolic pathways between the yolk and embryonic cells that may support anaerobic metabolism.

The general characterization of lactate as a poor anaerobic end-product may be unwarranted. In appropriate quantities, lactate has been seen to have important roles in regulating gene expression through epigenetic mechanisms. For example, lactate has been shown to inhibit histone deacetylase activity (HDAC), which can have genome wide effects (Latham et al., 2012). In addition, recent evidence suggests that histones can be post-translationally modified by the addition of lactate (lactylation) which induces change in gene expression through modification of chromatin structure (Zhang et al., 2019). Lactate has also been found to have a neuroprotective role, preventing excitotoxic cell death and

insults from cerebral ischemia (Berthet et al., 2009; Jourdain et al., 2016). It was recently shown that human neuroblastoma cells (SH-SY5Y) and *C. elegans* pretreated with lactate promoted oxidative stress resistance and resulted in long-term stress resistance and longevity (Tauffenberger et al., 2019). Thus, it is possible that lactate is a necessary and conserved part of the vertebrate response to anoxia, and when coupled with the proper mechanisms for metabolic suppression can be an effective means of supporting anaerobic metabolism for long-term survival.

Previous studies in Crucian carp suggest that GABA may play a role in suppressing metabolism during anoxia as indicated by a near three-fold increase in ethanol (the major anaerobic end-product) production when fish were injected with isoniazid, an inhibitor of GAD (Nilsson, 1992). In *A. limnaeus*, GABA synthesis and lactate production appear to be independent as evidenced by no change in lactate accumulation with inhibition of GAD, GABA-T, or SSADH. While it is possible that inhibition of GABA synthesis could be affecting some other major metabolic pathway such as ethanol production, it is unlikely given the rather extensive metabolomics studies performed on *A. limnaeus*, the generally low levels of transcripts for proteins in this pathway, and the fact that previous efforts have failed to detect ethanol production in embryos (Podrabsky and Hand, 1999). Thus, we conclude that GABA is not playing a general role in suppression of metabolism during anoxia in embryos of *A. limnaeus*.

Decreased survival when GABA-T and SSADH are inhibited suggests that at least part of the GABA produced during anoxia is being metabolized and

shuttled into the TCA cycle. Because GABA degradation can lead to ammonia consumption, NADH oxidation, and GTP production (Goldlust et al., 1995), this response could be beneficial for supporting embryonic metabolism during exposures to anoxia. The rate of lactate accumulation appears to decrease over long-term anoxia, which may indicate a shift in reliance to GABA metabolism.

Although GABA metabolism appears to be critical for long-term survival in anoxia, the innate capacity for high GAD activity does not correspond to anoxia tolerance. We hypothesized that embryos from anoxia-tolerant stages would have higher basal GAD enzymatic activity than anoxia-sensitive stages. However, we saw an inverse relationship between normoxic GAD activity and anoxia tolerance. Despite having the highest capacity to make GABA, WS 42 embryos produced negligible GABA in anoxia. This is contrary to what is seen with LDH capacity in *A. limnaeus* embryos, where there is an increase in LDH capacity with post-DII development that translates to high LDH activity during anoxia (Chennault and Podrabsky, 2010).

Anoxia-induced GABA synthesis is not unique to *A. limnaeus* embryos (Nilsson and Lutz, 2004; Nilsson et al., 1991; Podrabsky et al., 2007). Despite previous knowledge of GABA accumulation in anoxia, this is the first study to directly relate GABA metabolism to survival of *A. limnaeus* embryos in anoxia. We found that inhibition of enzymes responsible for both GABA synthesis (via GAD) and degradation (via GABA-T and SSADH) led to decreased survival (20-44% reduced LT₅₀s) in the most anoxia-tolerant developmental stage (WS 36). Inhibition of enzymes that degrade GABA (GABA-T and SSADH) lead to

increased accumulation of GABA, and decreased survival; thus, increased concentrations of GABA do not necessarily translate to longer survival. These data suggest a role for GABA as a metabolic intermediate supporting anoxic metabolism, and not a simple end-product. Interestingly, the effects of decreased GABA production on survival are not obvious until after 3–4 weeks of anoxia, suggesting a role in long-term rather than short-term survival. Further, doubling the dose of EKP to block GABA production had only a marginal effect on the LT₅₀, but resulted in a much more rapid time to 100% mortality. Thus, we conclude that GABA synthesis plays a critical role in long-term anoxia tolerance as a metabolic intermediate of critical importance.

The abundance and diversity of GABA transporters expressed in *A. limnaeus* embryos suggest GABA may be shuttled within the embryo or within cells after production. The expression of high levels of GABA transporters in DII embryos and anoxia-induced elevation in transcripts of GABA transporters in WS 36 embryos suggest an importance for GABA shuttling during anoxia. Several GABA transporters remain high or continue to increase during aerobic recovery in DII and WS 36 embryos. One possibility is that GABA is being transported into the yolk. Alternatively, GABA may be synthesized in the yolk and transported to the developing embryo. This is consistent with a lack of GABA production in two established anoxia-tolerant cell lines of *A. limnaeus* (PSU-AL-WS40NE, WS36-2 cells; unpublished data). Another possibility is that only specific cells within the embryo produce GABA, and the two neural-derived cell lines isolated are not responsible for GABA production. Nevertheless, preliminary evidence suggests

GABA present in both the yolk and cellular fractions of embryos (unpublished).

The internal pH of embryos and the localization of lactate and GABA during anoxia and recovery from anoxia are currently unknown and need to be resolved to fully understand the physiology of GABA production in this species.

The main function of GABA in adult vertebrates is as a neurotransmitter.

The diversity of GABA receptor subunits expressed in *A. limnaeus* embryos support the potential for GABA as an early neurotransmitter in this species.

GABA has been found to provide excitatory actions in the developing vertebrate nervous system, but conversely, typically functions as an inhibitory neurotransmitter in adults (Ben-Ari, 2002). The switch from excitatory to inhibitory transmission occurs due to delayed expression of K⁺-Cl⁻-coupled-co-transporters (KCC). Transcript expression data support GABA acting as an inhibitory transmitter during *A. limnaeus* development due to the rise in KCC transcripts as embryos develop to DII and beyond. This may represent a unique developmental adaptation to anoxia tolerance in *A. limnaeus*.

Increased GABA levels are thought to protect the brain of many organisms from excitotoxic cell death during hypoxic and anoxic conditions (Lutz and Milton, 2004; Lutz and Nilsson, 2004; Nilsson and Lutz, 2004; Podrabsky et al., 2007). A main factor that differentiates anoxia-tolerant and anoxia-sensitive species is their ability to maintain glutamate homeostasis in response to low oxygen levels (Nilsson and Renshaw, 2004). The ability to convert an excitatory neurotransmitter (glutamate) to an inhibitor neurotransmitter (GABA) may be beneficial to embryos trying to depress their metabolism and prevent excitotoxic

cell death during anoxia. Increased endogenous GABA release during anoxia dampened excitatory potentials in turtle brain (Pamenter et al., 2011) and preventing GABA reuptake and metabolism in mammalian models supports neuroprotection during ischemia (Prentice, 2009; Schwartz-Bloom and Sah, 2001). Furthermore, turtle neurons treated with GABA_A and GABA_B receptor antagonists exhibited seizure-like effects and eventual cell death, similar to excitotoxic cell death in anoxic mammalian brains (Pamenter et al., 2011).

Perhaps GABA is acting in a similar manner in *A. limnaeus*, thereby increasing the stimulation required to elicit an action potential. However, the accumulation of GABA in gross excess of that needed for neuroprotection in *A. limnaeus* (1000 times more than in other anoxia tolerant species), suggests multiple or novel functions. Localization of GABA receptors and transporters in embryos and their role in anoxia is worth further exploration. Functional tests through exposure of embryos to GABA receptor antagonists could lend insight into the neurotransmitter function of GABA in anoxia.

This is the first extensive study of long-term anoxia and aerobic recovery in *A. limnaeus*. Our findings suggest that embryos seemingly go unharmed and without developmental defect following long-term anoxia. However, recovery from anoxia appears to be slow with lactate and GABA levels not returning to pre-anoxia levels until after at least a week of aerobic recovery. This slow reduction of both lactate and GABA may be an adaptive and protective mechanism for supporting survival of long-term anoxia. A primary cause of mortality due to anoxic exposure is not the lack of oxygen itself, but ironically the

reoxygenation event that occurs after (Bundgaard et al., 2020; Saikumar et al., 1998). A rapid increase in reactive oxygen species (ROS) in response to reinitiating aerobic respiration can lead to damaging of DNA, cell membranes, and proteins (Schieber and Chandel, 2014). In anoxia-sensitive mammals, reoxygenation is associated with overproduction of ROS from mitochondria, which often initiates programmed cell death (apoptosis). Although ROS has not been quantified in embryos of *A. limnaeus*, they possess a large antioxidant capacity that is likely to help protect against ROS production (Wagner et al., 2019). A surge in ROS production can likely be minimized by a slow increase in aerobic metabolism following recovery. The inhibitory and sedative effects of GABA on neurons may prevent a surge of synapses firing, thereby decreasing ROS production by slowly increasing aerobic metabolism. This would be consistent with observations of a slow return of a heartrate in WS 36 embryos following long-term anoxia exposure. One interesting possibility is the shunting of GABA into polyamine metabolism during transitions from anoxia to normoxia, or perhaps during severe hypoxia. GABA can be converted to putrescine through a pathway that utilizes the enzyme diamine oxidase (DAO), which consumes hydrogen peroxide and produces molecular oxygen in the process. This would be an excellent method to help prevent accumulation of ROS damage during periods of severe oxygen limitation. The expression of genes involved in this pathway would support this possible function for GABA during recovery from anoxia or during prolonged severe hypoxia, both conditions where elevated hydrogen peroxide production would be expected. Because DAO requires

molecular oxygen or hydrogen peroxide to function, this pathway would be largely blocked during long-term anoxia allowing GABA to accumulate in preparation for use during periods of reoxygenation. In addition, this could be a mechanism for detoxification of ROS associated with episodic or brief reintroductions of oxygen. Consistent with this hypothesis, inhibition of DAO with amiloride has no effect on GABA accumulation during anoxia (Fig 2.6). Similarly, in the yeast *Saccharomyces cerevisiae*, the GABA shunt pathway has been shown to be critical in reducing ROS production and protecting against oxidative stress (Cao et al., 2013; Coleman et al., 2001). Though not yet investigated in animals, the conserved role of GABA across bacteria, yeast, plants, and animals in reduction of oxidative stress suggests this possibility in *A. limnaeus*. Perhaps GABA and lactate accumulated during anoxia aid in both surviving without oxygen and the reoxygenation event that follows. The combination of lactate and GABA serving as neuroprotective and antioxidant agents is worth exploring further.

Previous studies on *A. limnaeus* embryos have shown their extreme anoxia tolerance, (Meller et al., 2012; Podrabsky et al., 2007; Riggs and Podrabsky, 2017), but this is the first study to observe these metabolites in response to long-term anoxia across development and aerobic recovery. We show that GABA may have multiple functions in *A. limnaeus* embryos that could be contributing to survival during anoxia and recovery. Most importantly, we show that GABA is being synthesized and degraded using common pathways in vertebrates. Further exploration into the metabolism, signaling, and localization of

GABA and lactate in *A. limnaeus* embryos will provide further insight into how anoxia-sensitive organisms can survive anoxic events and avoid ischemic injury.

Table 2.1. Segmental linear regression analysis of GABA and lactate accumulation during exposure to anoxia

	Parameter	WS 32/33 (DII)	WS 36	WS 40	WS 42
GABA	Initial (mmol kg ⁻¹ embryo ⁻¹ d ⁻¹)	0.126	0.281	0.561	^a
	X ₀ (d)	16	18	2	^a
	Final (mmol kg ⁻¹ embryo ⁻¹ d ⁻¹)	0.035	0.156	0.733	^a
	Degrees of Freedom	16	16	13	^a
	R ²	0.97	0.97	0.96	^a
Lactate	Initial (mmol kg ⁻¹ embryo ⁻¹ d ⁻¹)	0.152	0.497	3.679	26.83
	X ₀ (d)	16	18	2	0.17
	Final (mmol kg ⁻¹ embryo ⁻¹ d ⁻¹)	0.090	0.253	2.088	3.322
	Degrees of Freedom	16	16	13	9
	R ²	0.80	0.97	0.95	0.96

^a GABA was barely detectable in WS 42 embryos and did not accumulate significantly (Regression analysis, $m = 0.124$, $R^2 = 0.134$, $P = 0.242$).

Figure 2.1. GABA and lactate accumulation in embryos of *Austrofundulus limnaeus* during exposure to anoxia and aerobic recovery from anoxia at 25°C. (A) A schematic showing the experimental design and sampling regimen used. Embryos from 4 developmental stages were sampled at their lethal time to 50% mortality (LT_{50}) in anoxia, and at the $LT_{50}/2$ and $LT_{50}/4$ (Meller et al., 2012; Podrabsky et al., 2007; Riggs and Podrabsky, 2017). Following exposure to anoxia, embryos were sampled during aerobic recovery from anoxia for up to 14 d. WS 42 embryos did not recover from anoxia and thus samples were not collected. DII embryos (WS 32/33) remained in diapause during anoxia, whereas WS 36 and WS 40 embryos resumed development. Arrow colors match the colors used in subsequent figures for each developmental stage. WS, Wourms' stage; DII, diapause II. (B) Accumulation of lactate and GABA during anoxia and aerobic recovery from anoxia. Vertical dotted lines indicate the LT_{50} of each developmental stage. Closed symbols indicate time points in anoxia while open symbols indicate timepoints during aerobic recovery. Each replicate consists of 5 embryos. Symbols represent means $\pm$ SEM ($N = 4$ for DII, WS 36, and WS 40; $N = 3$ for WS 42). Analysis of variance (ANOVA, $P < 0.0001$) indicates a significant accumulation of GABA and lactate for DII, WS 36 and WS 40 embryos, while only lactate accumulated in WS 42 embryos. Asterisks indicate the first timepoints in recovery that are not significantly different from $t = 0$ (Dunnett's *post hoc*, $P < 0.05$). Rates of GABA and lactate accumulation were calculated from the slopes of metabolite concentrations during exposure to anoxia. For each developmental stage, initial and final slopes were calculated using segmental linear regression. See Table 2.1 for results of regression analyses.

Figure 2.2. The relationship between lethal time to 50% mortality (LT_{50}) and anoxic GABA and lactate accumulation in embryos of *A. limnaeus*. (A) LT_{50} was highly correlated with initial rates of lactate accumulation in anoxia in embryos of *A. limnaeus* (Correlation analysis, $P < 0.05$). When initial rates of lactate accumulation of early developing embryos from Podrabsky et al. (2007) are included, the relationship was strengthened (Correlation analysis, $P < 0.0001$). Slopes from Podrabsky et al. (2007) and data presented here are similar (ANCOVA, $P = 0.1886$). Final rates of lactate accumulation were not significantly correlated with survival (Correlation analysis, $P = 0.0940$). Each replicate consists of 5 embryos. Symbols represent means $\pm$ SEM ($N = 4$ for DII, WS 36, and WS 40; $N = 3$ for WS 42). Error bars for certain stages are within the symbol. (B) There was no correlation found between LT_{50} and rates of GABA accumulation (Correlation analysis $P > 0.05$). (C) There was a negative relationship between LT_{50} and the ratio of lactate to GABA accumulated during anoxia (Correlation analysis, $P < 0.05$). Values were determined by dividing the total lactate by total GABA accumulated at each stage's respective LT_{50} for each biological replicate. Note that x- and y-axes are on log scale for all panels.

Figure 2.3. Survival of embryos to 800 $\mu\text{mol l}^{-1}$ ethyl ketopentenoate (EKP) in normoxia and anoxia. Wourms' stage (WS) 36 embryos exposed to EKP in anoxia survived longer than developing embryos (WS 36 and WS 40) exposed to EKP in normoxia. WS 40 embryos exhibited mortality after 2 d of exposure, whereas WS 36 embryos exhibited mortality after 4 d. Vertical dotted lines indicate completion of embryological development. WS 36 embryos in anoxia remained at WS 36 for the duration of EKP exposure. Filled symbols represent percent survival of 15 individually monitored embryos in normoxia. Open symbols represent mean survival $\pm$ SEM of groups of 15 embryos ($N = 3$) and are from Figure 2.4 and are provided here for ease of comparison.

Figure 2.4. GABA and lactate accumulation during inhibition of the GABA synthesis and degradation pathway in Wourms' stage 36 embryos exposed to anoxia. (A) A schematic depicting canonical and non-canonical synthesis and metabolic degradation of GABA and the key enzymes pharmacologically inhibited in experiments. GAD, glutamate decarboxylase; GABA-T, GABA transaminase; SSAL, succinic semialdehyde; SSADH, succinic acid semialdehyde dehydrogenase; EKP, ethyl ketopentenoate; DEAB, N,N-diethylaminobenzaldehyde; ALDH9A1, aldehyde dehydrogenase; DAO, diamine oxidase; ODC, ornithine decarboxylase; SAT, spermidine/spermine N1-acetyltransferase; PAOX, polyamine oxidase. (B,C) GABA accumulated in a linear manner for the control, EKP, DEAB, and vigabatrin treatments (Regression analysis, $P < 0.0001$). Rates of GABA accumulation (m) for all treatments and the controls were significantly different from one another (B: ANCOVA, $F = 55.96, P < 0.0001$; C: ANCOVA, $F = 74.54, P < 0.0001$). Asterisks indicate treatment timepoints that were significantly different from the control (two-way ANOVA with Tukey's HSD, $P < 0.05$). Symbols represent means $\pm$ SEM ($N = 3$) and each replicate contained 5 embryos. (D,E) Lactate accumulated in a linear manner for the control, EKP, DEAB, and vigabatrin treatments (Regression analysis, $P < 0.0001$). Rates of lactate accumulation (m) were similar for the controls and all treatments (D: ANCOVA, $F = 1.750, P = 0.19$; E: ANCOVA, $F = 1.043, P = 0.36$). No timepoints for embryos treated with EKP, DEAB, or vigabatrin were significantly different from one another or the control (two-way ANOVA with Tukey's HSD, $P > 0.05$).

Figure 2.5. Survival of Wourms' stage 36 embryos continuously exposed to GAD inhibitor EKP, SSADH inhibitor DEAB, and GABA-T inhibitor vigabatrin in anoxia. Groups of 15 embryos were exposed to embryo medium containing (A) 400 $\mu\text{mol l}^{-1}$ EKP or 167 $\mu\text{mol l}^{-1}$ DEAB, (B) 800 $\mu\text{mol l}^{-1}$ EKP, or 1 mmol l^{-1} vigabatrin in anoxia at 25°C. Symbols are means $\pm$ SEM ($N = 3$). (C) Lethal time to 50% mortality (LT₅₀) for embryos exposed to anoxia. Different letters indicate significant differences in LT₅₀ between treatments (one-way ANOVA with Tukey's HSD test, $P < 0.05$). Bars are means $\pm$ SEM (Control, $N = 6$; Inhibitors, $N = 3$) and symbols represent individual replicates. GAD, glutamate decarboxylase; EKP, ethyl ketopentenoate; SSADH, succinic acid semialdehyde dehydrogenase; DEAB, N,N-diethylaminobenzaldehyde; GABA-T, GABA transaminase.

Figure 2.6. GABA accumulation during inhibition of diamine oxidase (DAO) by amiloride in *Wourms' stage 36 embryos* exposed to anoxia. Rates of GABA accumulation (m) were similar for the control and amiloride treatments (ANCOVA, $F = 0.2208$, $P = 0.65$). There was no significant difference in GABA accumulated after 8 d (unpaired t-test, $P = 0.64$). Symbols represent means $\pm \text{SEM}$ ($N = 4$) and each replicate contained 5 embryos. Slopes (m) were calculated using linear regression analysis.

Figure 2.7. Glutamate decarboxylase (GAD) activity in adult male *A. limnaeus* brain homogenates in normoxia at 25°C. Adult brain homogenates generate GABA that are significantly greater than 0 at pH 7.0, 8.0, and 9.0 (Regression analysis, $P < 0.005$), while pH 6.0 homogenates did not show a significant accumulation of GABA after 3 h of incubation (Regression analysis, $P = 0.1865$). Rates of GABA production and thus GAD activity were based on the slope (m) of GABA accumulation over time. Symbols are individual replicates. All replicates between pH treatments originated from the same brain homogenate.

Figure 2.8. Glutamate decarboxylase (GAD) activity in *A. limnaeus* embryo homogenates across development in normoxia at 25°C. (A) Post-DII embryo homogenates generate GABA at low rates that are significantly greater than 0 (Regression analysis, $P < 0.01$) while DII embryo homogenates did not show a significant accumulation of GABA after 24 h of incubation (Regression analysis, $P = 0.1701$). Rates of GABA production and thus GAD activity were based on the slope (m) of GABA accumulation over time. Symbols are means $\pm$ SEM ($N = 4$). Each replicate consisted of 13 embryos. (B) Predicted initial GABA accumulation based on GAD activity compared to observed GABA accumulated in embryos exposed to anoxia (from Fig 2.1B). Despite not accumulating significant amounts of GABA during anoxia, observed mean GABA levels are included for WS 42 embryos. Different letters indicate significant differences in predicted and observed values within developmental stage (unpaired t-test, $P < 0.05$). (C) There is a negative relationship between LT₅₀ and GAD activity in embryos of *A. limnaeus*. Exponential one-phase decay analysis provides a good fit for the two variables ($R^2 = 0.96$). Symbols represent means $\pm$ SEM ($N = 4$).

Figure 2.9. Transcript levels for GABA, polyamine, lactate, and ethanol pathways in *A. limnaeus* as determined by RNA sequencing. Enzyme systems are organized into groups: (A) GABA synthesis and degradation, (B) polyamine metabolism, (C) lactate and ethanol synthesis, and (D) GABA and lactate transportation (WS 20–31) were obtained from embryos incubated at 20°C and were collected at the developmental stages shown at the bottom of the heatmap. Embryos in DII and post-DII were incubated at 25°C and timepoints for anoxia and aerobic recovery are shown below the heatmap: 0, normoxia; 4A, 4 h anoxia; 24A, 24 h anoxia; 4R, 4 h recovery; 24R, 24 h recovery; 2A, 2 h anoxia; 2R, 2 h recovery. Expression levels are reported as mean fragments per kilobase per million mapped reads (FPKM). $N = 3$ for pre-DII stages; $N = 4$ for DII, WS 36, and WS 42; $N = 6$ for WS 40. WS, Wourms' stage. Cells in yellow indicate transcript levels that were overexpressed (FPKM = 11,000–30,000).

Figure 2.10. Transcript levels for GABA signaling and cotransporters in *A. limnaeus* as determined by RNA sequencing. Enzyme systems are organized into groups: GABA_A receptors and subunits, GABA_B receptors and subunits, GABA receptor associated proteins, and Na⁺-K⁺-Cl⁻ (NKCC) and K⁺-Cl⁻-cotransporters (KCC). Data for pre-DII development (WS 20–31) were obtained from embryos incubated at 20°C and were collected at the developmental stages shown at the bottom of the heatmap. Embryos in DII and post-DII were incubated at 25°C and timepoints for anoxia and aerobic recovery are shown below the heatmap: 0, normoxia; 4A, 4 h anoxia; 24A, 24 h anoxia; 4R, 4 h recovery; 24R, 24 h recovery; 2A, 2 h anoxia; 2R, 2 h recovery. Expression levels are reported as mean fragments per kilobase per million mapped reads (FPKM). *N* = 3 for pre-DII stages; *N* = 4 for DII, WS 36, and WS 42; *N* = 6 for WS 40. WS, Wourms' stage.

Figure 2.11. The relationship between female age and rates of GABA and lactate accumulation in anoxic Wourms' stage 36 embryos. (A) The rate of GABA accumulation in embryos did not correlate with female age; however (B) there was a significant negative linear relationship in the rate of lactate accumulation with female age. Each symbol represents a single rate of accumulation calculated from biological replicates ($N = 3-4$) sampled up to 30 d in anoxia.

Chapter 3

No water, no problem: Stage-specific metabolic responses to dehydration stress in annual killifish embryos

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Introduction

Water is essential for life and the threat of dehydration due to evaporative water loss is ever-present in terrestrial organisms and especially in aquatic organisms that exploit terrestrial environments (Martin and Cooper, 1972; Martin and Carter, 2013; Martin et al., 2004; Martin, 1999; Moravek and Martin, 2011). In all terrestrial organisms, respiratory gas exchange and water loss are closely related: if oxygen and carbon dioxide can be exchanged, so can water vapor (Danks, 2000). Subsequently, nearly all terrestrial animals have evolved mechanisms to dramatically reduce loss of water through the integument and support efficient gas exchange through specialized respiratory organs. Those animals that do rely on cutaneous gas exchange must remain in moist or semi-aquatic habitats (e.g., salamanders and frogs). Even fewer aquatic organisms can exploit terrestrial habitats for prolonged periods of time. Annual killifishes exploit ephemeral pond habitats that can be devoid of liquid water for several

months and perhaps even years (Polačik and Podrabsky, 2015). Survival of prolonged water stress is invested solely in drought-tolerant embryos that survive in the soil of dry ponds. Despite the clear importance of dehydration tolerance to the survival of annual killifishes, many questions remain regarding how long embryos can survive without water, their metabolic rate during aerial exposure, and the molecular mechanisms that support survival.

Many fish embryos that experience aerial conditions are found in moist environments, therefore evading severe desiccation (Martin, 1999; McDowall and Charteris, 2006; Podrabsky et al., 2010b; Taylor, 1999; Tingaud-Sequeira et al., 2009; Wells et al., 2015). The duration spent in the aerial environment for many species is usually short, on the magnitude of days or weeks (Martin et al., 2011; Thompson et al., 2017). The annual killifishes are unique exceptions in that they regularly endure long periods (months) of no water. The annual killifish, *Austrofundulus limnaeus*, is an emerging model for developmental physiology and ecology because of their wide array of environmental stress tolerances, annotated genome, and the ability to manipulate developmental trajectories in the lab (Podrabsky et al., 2017; Podrabsky et al., 2010a; Romney et al., 2018; Wagner et al., 2018). Adult *A. limnaeus* live in ephemeral ponds in the coastal deserts of Venezuela and their embryos have the remarkable ability to tolerate anoxic and dehydrating conditions for months (Podrabsky et al., 2016). Survival is attributed to the ability of these embryos to enter a state of profound metabolic dormancy (diapause) as a part of their normal development (Podrabsky and Hand, 1999; Podrabsky and Hand, 2000). Embryos can enter diapause at three

distinct stages termed diapause I, II, and III (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c). In *A. limnaeus*, diapause II (DII) is the most stress-tolerant stage and is likely responsible for long-term survival of dehydrating conditions (Podrabsky et al., 2016; Podrabsky et al., 2001; Polačik et al., 2020).

Embryos of *A. limnaeus* lose water in the first few days of aerial exposure, but after about a week the rate of water loss approaches zero (Podrabsky et al., 2001). Importantly, most of this water is lost from the extraembryonic perivitelline compartment, while the embryo appears to remain fully hydrated. Dramatically reduced permeability to water vapor suggests highly limited gas exchange in general, and thus we hypothesize that this mechanism for survival of dehydration stress may result in lower respiratory gas exchange and produce a self-imposed hypoxia or anoxia. Lack of oxygen (anoxia) is known to induce quiescence (halting development) in developing embryos of *A. limnaeus* (Podrabsky et al., 2007), and thus we also predict that dehydration may directly or indirectly induce developmental arrest.

Here we report on the survival and metabolic responses of dormant (DII) and actively developing post-diapause II embryos under aerial conditions to evaluate how long these embryos might survive water stress. We evaluate these data in relation to the ecology of embryonic development in annual killifishes. These data support a stage-specific response to dehydration stress that leads to increased variation in developmental rate and presumably physiological phenotypes in late-stage embryos only after they exit diapause II. Thus, long-term survival in the soil may be supported by entrance into diapause II while

intrinsic variation in developmental rate in response to a mixture of dehydration and oxygen stress may lead to a variety of developmental outcomes.

Materials and Methods

Animal husbandry and embryo collection

Adult annual killifish were housed and embryos collected as previously described (Podrabsky, 1999). This work was performed under established protocols which were approved and reviewed by the Portland State University Institutional Animal Care and Use Committee (PSU IACUC protocols #33 and 64). Briefly, adult annual killifish were kept in male-female pairs and spawned semiweekly. Embryos were collected and stored at 25°C with no light in 15 x 100 mm plastic Petri dishes in media that resembles the environmental conditions from which adults were collected in 1995 (10 mmol l⁻¹ NaCl, 2.15 mmol l⁻¹ MgCl₂, 0.8 mmol l⁻¹ CaCl₂, 0.14 mmol l⁻¹ KCl, 1.3 mmol l⁻¹ MgSO₄) (Podrabsky, 1999; Podrabsky et al., 1998). During the first 4 days post-fertilization (dpf), embryo medium contained methylene blue (0.0001%) to prevent fungal infection. Embryos were then treated with two 5 min washes of a solution of 0.01% sodium hypochlorite (separated by a 5 min rest in embryo medium) to prevent bacterial and fungal growth, as previously described (Podrabsky, 1999). Following sodium hypochlorite treatment, embryos were transferred to embryo medium containing 10 mg l⁻¹ gentamicin sulfate and allowed to develop to DII (32–46 d). To break DII, embryos were subjected to a temperature of 30°C and full spectrum light for 48 h (Meller et al., 2012). Following this treatment, embryos were sorted into

synchronized cohorts of embryos by developmental stage (Podrabsky et al., 2017).

Embryonic stages investigated

Experiments were performed on four developmental stages of embryos (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c) to capture a gradient of stress tolerance levels and physiology: Wourms' stage (WS) 32/33 or diapause II (DII), WS 36 (4 days post-diapause II (dpd)), WS 40 (12 dpd), and WS 42 (20 dpd). DII embryos are metabolically dormant and have halted development, whereas the other stages are metabolically active and developing.

Tolerance of aerial exposure

Embryos (DII, WS 36, WS 40, WS 42) were exposed to 85% relative humidity (RH) air at 25°C with no light in a sealed plastic desiccator (Nalgene, 250 mm diameter). Relative humidity was controlled by using a saturated solution of potassium chloride (750 ml) placed below the shelf and continually mixed with a stir bar to ensure uniform RH within the aerial portion of the chamber (Podrabsky et al., 2001; Winston and Bates, 1960). Prior to dehydration exposure, embryos were treated with sodium hypochlorite (see above) and were allowed to recover for 2 h at 25°C without light. Groups of 20 embryos ($N = 3$) per stage were removed from their aqueous medium and placed in Petri dishes (50 mm, PDF2047S0, Fisher Scientific, Hampton, NH, USA) containing a sterilized filter pad saturated with 2.5 ml of embryo medium containing 10 mg l⁻¹ gentamicin

sulfate (Podrabsky et al., 2001). Embryos were monitored until 100% mortality was reached. Embryos were determined dead when they lost their shape and shine. Survival between stages was compared by calculating lethal time to 50% mortality (LT_{50}) in response to aerial exposure.

Metabolic rate (respirometry)

Aerial incubation

Diapause II. Embryos were treated with sodium hypochlorite (see above) and incubated in embryo medium containing 10 mg l⁻¹ gentamicin sulfate and 2.5 µg l⁻¹ amphotericin B (10128-872, VWR, Radnor, PA, USA) for 24 h prior to aerial exposure. For aerial incubation, DII embryos were placed on filter pads containing 2.5 ml embryo medium containing gentamicin and amphotericin B using sterile technique within a biosafety cabinet. Care was taken to make certain that single embryos were isolated and not touching other embryos. DII embryos were then exposed to 85% RH at 25°C without light for 11 d to allow equilibration of the filter pad with the aerial environment. Four biological replicates ($N = 4$), comprised of five embryos each, were placed in standard opening 2 ml borosilicate glass crimp-top vials (03-391-2, 12 OD x 32 mm long, Fisher Scientific, Hampton, NH, USA) containing a 4 mm RedEye oxygen sensor patch for non-invasive oxygen monitoring (Ocean Insight, Largo, FL, USA). Transfer of embryos was done within a biosafety cabinet using sterile technique. Vials containing embryos and sensor patches were crimped with aluminum crimp seals and a black Viton™ elastomer septa (03-378-343, Thermo Scientific,

Waltham, MA, USA). Vials and septa were equilibrated at 85% RH for 30 min prior to use. To ensure sterilization of vials and sensor patches, vials containing patches were prepared in a biosafety cabinet and UV-sterilized for several hours prior to addition of embryos.

Wourms' stage 36 embryos. Diapause II embryos were treated with sodium hypochlorite and incubated in embryo medium containing 10 mg l⁻¹ gentamicin sulfate and 2.5 µg l⁻¹ amphotericin B for 24 h. Embryos were placed on filter pads containing 2.5 ml embryo medium with gentamicin and amphotericin B using sterile technique and placed into a desiccator at 85% RH (see details above). Diapause II was broken under aerial incubation by placing the desiccator under full spectrum light in an incubator at 30°C. After 72 h, temperature was reduced to 25°C and the light was turned off. Following 24 h of 25°C without light, embryos were removed and staged. Filter pads were then weighed to ensure equilibration with chamber relative humidity and showed no difference with the filter pads used for DII treatments. For WS 36 embryos, 7 biological replicates ($N = 7$), comprised of five embryos each were used. Embryos were added to 2 ml vials containing sensor patches and crimp sealed under sterile conditions as described above.

Aqueous incubation

Diapause II and WS 36 embryos were sorted and treated with sodium hypochlorite as described above. Following treatment, embryos were allowed to recover for 2 h prior to the start of the respirometry experiment. Embryo medium

containing 10 mg l⁻¹ gentamicin sulfate was aerated by gentle bubbling with atmospheric air at 25°C for 1 h to ensure dissolved oxygen (DO) saturation. To prevent supersaturation, medium was allowed to equilibrate for 5 min without aeration prior to use. Embryo medium was then added to vials containing 5 embryos each (DII, *N* = 4; WS 36, *N* = 5) and a sensor patch. Vials were immediately crimp sealed with Viton™ caps making sure to leave no air bubbles trapped within the vials.

Oxygen measurement and embryo observation

Measurements of sensor patch fluorescence were made through the vial wall using a fiber optic probe in conjunction with a NeoFox Oxygen Sensing System (Ocean Insight, Largo, FL, USA). For aqueous conditions, the system was calibrated to 100% DO saturation using vials containing embryo medium aerated with atmospheric air at 25°C and 0% DO saturation using embryo medium bubbled with N₂ for 30 min and equilibrated overnight in a Bactron III anaerobic chamber (Sheldon Manufacturing, Cornelius, OR, USA), containing 5% CO₂, 5% H₂, and 90% N₂. For aerial conditions, the system was calibrated to 100% saturation using vials containing atmospheric air at 25°C and 0% saturation using 5% CO₂, 5% H₂, and 90% N₂ from the anaerobic chamber. Previous observations illustrated no effects of the anaerobic gas mixture on fluorescence measurements (data not shown). Vials sealed at 0% and 100% saturation (both aerial and aqueous) were regularly measured alongside samples. These control vials remained consistent across the entire duration of

the experiments, and were used as blank vials to control for the possibility of contamination and to ensure stable calibration of the NeoFox Oxygen Sensing System. Oxygen concentration was monitored regularly and rate of oxygen consumption was used as a proxy for metabolic rate. All measurements were taken at 25°C with minimal light. Following measurement of oxygen concentration, embryos were observed under a dissection microscope (Leica, S8 APO, Wetzlar, Germany) to record health and developmental stage. Rate of oxygen consumption was calculated using the slope of decrease in oxygen content inside each vial over time divided by the number of embryos in the vial. Oxygen consumption is presented as $\text{nmol O}_2 \text{ h}^{-1} \text{ embryo}^{-1}$.

Statistical analysis

Graphical and statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA, USA) and SPSS software (IBM., v.26.0, Armonk, NY, USA). LT_{50} of embryos exposed to dehydration was calculated for each developmental stage by probit regression analysis (Chapman et al., 1995) using SPSS software. Where appropriate, analysis of variance (ANOVA), unpaired t-test, or linear regression analyses were used. Tukey's honest significant different (HSD) test was used for *post-hoc* comparisons, where applicable. Statistical significance was set to $P < 0.05$ for all comparisons.

Results

Tolerance of aerial exposure

Embryos of *A. limnaeus* exhibited a profound tolerance of aerial conditions (85% RH). This tolerance peaked in DII embryos, with 10% surviving over 500 d and a single embryo living for 587 d (Fig. 3.1A). Remarkably, all developmental stages had embryos survive longer than 100 d. When the survival data were used to calculate lethal time to 50% mortality (LT_{50}), DII embryos exhibited an LT_{50} of 325 ± 17 d (mean $\pm$ SEM), which was significantly higher than the post-DII stages (Fig. 3.1B, one-way ANOVA with Tukey's HSD, $P < 0.0001$). Wourms' stage 36 embryos exhibited an LT_{50} of 84 ± 5 d, which was significantly higher than later stage embryos which had similar LT_{50} values of 28 ± 6 d (WS 40) and 29 ± 5 d (WS 42) (Fig. 3.1B, one-way ANOVA with Tukey's HSD, $P < 0.05$). Embryos were not observed under a microscope, but macroscopic observation showed no eye development nor pigmentation in DII embryos, suggesting that embryos remained in diapause for the duration of the experiment.

Metabolic rate

Diapause II

Diapause II embryos consumed available oxygen in aqueous vials faster than aerial vials (Fig. 3.2A). However, because total oxygen available was 33 times lower in water compared to air, aerial exposed embryos consumed more available oxygen per unit time than aqueous exposed embryos (Fig 3.2C). Oxygen decreased in a linear fashion in trials using DII embryos, and thus a

single linear regression was performed on each replicate to calculate the overall metabolic rate. To calculate rate of oxygen consumption, linear regressions were performed for each biological replicate ($N = 4$) for aerial and aqueous treatments (Fig 3.2E; Table 3.1). Rates of oxygen consumption for DII embryos reported here (Fig 3.3A; $0.15 \pm 0.01 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$, mean $\pm$ SEM, $N = 4$) are similar (unpaired t-test, $P = 0.73$) to those from previous experiments ($0.14 \pm 0.01 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$, mean $\pm$ SEM, $N = 4$ (Podrabsky and Hand, 1999)). Diapause II embryos exposed to aerial conditions had a rate of oxygen consumption that was over 4 times higher than those exposed to aqueous conditions (Fig 3.3A, Table 3.1; unpaired t-test, $P = 0.0005$). Diapause II embryos remained in diapause for the duration of the experiment in both aerial and aqueous treatments.

Post-diapause II (Wourms' stage 36)

All embryos began respirometry experiments at Wourms' stage 36. Initial rates of oxygen consumption in WS 36 embryos are similar in aerial versus aqueous conditions (Fig 3.3B; one-way ANOVA with Tukey's HSD test, $P = 0.9999$). Mean rates of oxygen consumption for aqueous ($1.2 \pm 0.04 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$) and aerial ($2.3 \pm 0.41 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$) WS 36 embryos reported here are also similar (ANOVA, $P = 0.07$) to those reported previously for aqueous embryos ($1.6 \pm 0.25 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$ (Podrabsky and Hand, 1999)). The duration of the aqueous exposures in WS 36 embryos was short due to exhaustion of oxygen in the vials – nearly all oxygen was consumed after 72 h (Fig 3.2B). For aqueous exposures, a single linear regression was performed for

the initial 72 h of each replicate (Fig 3.2F; Table 3.1). Post-diapause II embryos continued to develop at different rates during aerial exposures (Fig 3.3C) leading to a divergence in oxygen consumption rates between replicates over time (Fig 3.2F, 3.3C). For aerial incubations there was a clear initial rate of oxygen consumption early in the treatments that transitioned to much higher rates later in the trial, and thus two linear regressions were performed on each vial to maximize R squared values for initial (WS 36) and final (WS 38-42) rates of oxygen consumption (Fig 3.2F; Table 3.1). For aerial embryos, initial oxygen consumption includes the first 69 h and final rates were calculated using data between 165 and 452 h. The timepoints between 69 h and 165 h were omitted from the regression analyses because oxygen content did change in a linear fashion. Time points after 452 h were omitted because oxygen content in the vials was below the measured critical oxygen level (P_{crit}) of *A. limnaeus* late-stage embryos (Podrabsky and Wilson, 2016). Interestingly, there was a noticeable difference in the amount of oxygen remaining in each replicate vial in the aerial treatment, ranging from 4 to 13 μmol per vial. However, long-term aerial incubated embryos had a significantly higher rate of oxygen consumption than initial rates observed in aerial and aqueous treatments, as would be expected for embryos that continue to develop (Fig 3.3B; one-way ANOVA with Tukey's HSD test, $P < 0.01$). Mean rates of oxygen consumption in aerial WS 42 embryos that developed synchronously and continuously (the first 3 vials in Fig 3.3C; $6.14 \pm 0.12 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$) were significantly lower (unpaired t-test, P

= 0.003) compared to those reported from previous studies ($16 \pm 1.5 \text{ nmol hr}^{-1} \text{ embryo}^{-1}$ (Podrabsky and Hand, 1999)).

Discussion

Despite knowledge of the unprecedented ability of annual killifish embryos to survive long periods of aerial exposure, this is the first time that LT_{50} calculated values have been measured under controlled conditions across development. The ability of these embryos to survive in 85% RH for hundreds of days is impressive for an aquatic embryo especially considering that 85% RH is equivalent to being bathed in a solution of around $8000 \text{ mOsmol kg}^{-1}$ salt (around 8X sea water). This ability is almost unparalleled in any other aquatic species and certainly suggests unique adaptations for preventing evaporative water loss. In addition, this is the first time that aerial oxygen consumption has been measured in *A. limnaeus* embryos. We hypothesized that aerial incubation would limit gas exchange with the environment in concert with the observed decrease in evaporative water loss (Podrabsky et al., 2001) and lead to self-imposed hypoxia or anoxia. While results for late-stage embryos are consistent with this hypothesis, DII embryos respond in an opposite manner. This leads to an especially interesting question of how these embryos increase their rate of oxygen consumption while simultaneously reducing evaporative water loss to near zero and supporting survival for over a year under extremely dehydrating conditions. These unique responses to dehydration in dormant versus developing embryos may have important implications for the ecology of embryos in the wild.

Rates of oxygen consumption reported here for aqueous conditions are similar to those reported for embryos of *A. limnaeus* from previous experiments (Podrabsky and Hand, 1999), despite the use of different respirometry techniques. Interestingly, when exposed to aerial conditions, DII embryos have a higher rate of oxygen consumption compared to aqueous conditions. This is inconsistent with data from a species of non-annual killifish, *Kryptolebias marmoratus*, which exhibits a decrease in oxygen consumption under aerial incubation (Wells et al., 2015). These results were even more surprising given that even under aerobic aqueous conditions, DII embryos appear to support a large portion of their metabolism via anaerobic pathways (Podrabsky and Hand, 1999; Podrabsky et al., 2012a). Embryos incubated in aerial conditions did not break diapause and continue developing, which suggests either an energetic cost of survival of aerial conditions, or a diffusion-limited metabolism under aqueous conditions.

Annual killifish embryos, like all fish embryos prior to hatching, are limited to diffusive gas exchange with their environment. Under conditions of stagnant water flow, this can lead to impressively large boundary layers (up to 5 mm) that may limit rates of diffusion (DiMichele and Powers, 1984). Further, annual killifishes possess an egg envelope that lacks pores and is thicker than in most species of fish (Schoots et al., 1982), which may also contribute to limited rates of diffusion. Thus, perhaps the greater oxygen consumption observed in aerial DII embryos is due to improved rates of gaseous diffusion in an aerial environment as a consequence of a reduced effective boundary layer and the

greater availability of oxygen in air compared to water. When exposed to aerial conditions, embryos of *A. limnaeus* lose most of the water in the perivitelline space within the first week (Podrabsky et al., 2001), which effectively brings the egg envelope closer to the fully hydrated embryonic compartment and may further reduce the diffusion distance for gas exchange. The diffusion coefficients through the egg envelope under hydrated and aerial conditions are unknown and would need to be determined to evaluate if diffusion may be limiting gas exchange in DII embryos under aqueous conditions. However, this explanation is unlikely given data from other species (DiMichele and Powers, 1984) and the extremely low metabolic rates for DII embryos compared to post-diapause II embryos of *A. limnaeus* that do not exhibit an increase in oxygen consumption under aerial conditions. Thus, it is much more likely that the increased rate of oxygen consumption in DII embryos represents the cost of a physiological response needed for survival of dehydrating conditions.

The long-term survival of DII embryos during aerial incubation (over a year) may require physiological responses to evaporative water loss such as membrane restructuring, accumulation of metabolites, and alteration of compounds in the perivitelline space. All of these responses would likely increase metabolic demand. The egg envelope, perivitelline space, and enveloping cell layer of annual killifish embryos likely work in concert to resist evaporative water loss and support survival of aerial conditions (Podrabsky et al., 2001) and molecular changes to these structures are of interest in explaining the extreme dehydration tolerance and lack of water loss over time. This could require the

synthesis and accumulation of hydrophobic compounds in the membranes and perivitelline space, as suggested by Podrabsky et al. (2001). The soil dwelling collembolan *Folsomia candida* combats desiccation with a large increase in membrane mono-unsaturated fatty acids coupled with an increase of compatible osmolytes such as myo-inositol and glucose (Bayley and Holmstrup, 1999; Bayley et al., 2001). Similar observations were made in the Antarctic midge, *Belgica antarctica*, where dehydration led to increased fatty acid desaturase (FAD) gene expression, suggesting membrane restructuring via unsaturation (Lopez-Martinez et al., 2009). An increase in lipid content, especially neutral lipids, may be a mechanism to create lower resistance routes for oxygen diffusion into the embryo due to oxygen's higher solubility in lipids compared to water (Sidell, 1998). Unfortunately, little is known at the metabolic level in *A. limnaeus* embryos during aerial exposure, but results from this study suggest active mechanisms that support survival of long-term dehydration stress.

Initially, post-DII embryos (WS 36) of *A. limnaeus* maintained a similar rate of oxygen consumption in aerial and aqueous conditions. This result is consistent with previous reports for embryos of the non-annual killifish *Fundulus heteroclitus* (DiMichele and Powers, 1984; Tingaud-Sequeira et al., 2009). In contrast, WS 42 embryos of *A. limnaeus* have rates of oxygen consumption under aerial conditions that are over 60% lower than embryos under aqueous conditions. This result for late stage embryos is consistent with observations in *K. marmoratus* which experience a 44% reduction in oxygen consumption in response to aerial exposure (Wells et al., 2015). The differences in metabolic responses during

initial and long-term dehydration for *A. limnaeus* is intriguing and may be due to developmental differences in response to dehydration stress, or to the experimental conditions used. For instance, in the present study, embryos were allowed to develop until DII under aqueous conditions and then transitioned into aerial incubation as diapause was broken, while for *K. marmoratus* the embryos experienced aerial conditions for the entirety of development. It is important to note that developmental rates of *A. limnaeus* begin to diverge at relatively high levels of oxygen that are above the measured P_{crit} of *A. limnaeus* embryos under aqueous incubation conditions (Podrabsky and Wilson, 2016). Thus, lower rates of oxygen consumption are not likely due to an acute response to hypoxia, but more likely represent a physiological response to a mixture of dehydration and hypoxia.

In contrast to other species of fish examined which tend to develop synchronously under aerial conditions (DiMichele and Powers, 1984; Tingaud-Sequeira et al., 2009; Wells et al., 2015), development in *A. limnaeus* tends to be asynchronous after a week of aerial incubation. While the majority of the embryos continued developing similar to their aqueous counterparts, a subset of the embryos in four of the experimental vials remained at WS 38 from day 7 until the completion of the experiment 12 d later. Embryos of *A. limnaeus* enter quiescence in response to anoxia (Podrabsky et al., 2007), but this is the first report of quiescence-like behavior in this species in response to aerial conditions. It is likely that this stalling of development may be due to a combination of mild hypoxia and aerial exposure, because embryos exposed to 85% RH in large

containers where oxygen is not limited tend to develop synchronously (D.E.Z., personal observation). Of great interest is the apparent intrinsic variation observed in response to the aerial incubation conditions, which leads to developmental arrest in only a proportion of embryos.

The asynchronous development observed in post-DII embryos of *A. limnaeus* in response to aerial incubation could have profound influences on the developmental ecology of this species. Creating a pool of embryos in the soil at different developmental stages could be advantageous for persistence of the species by reducing the probability of total recruitment failure (Polačik et al., 2020). In contrast, synchronous development may lead to all embryos hastily responding to the same cue in a similar manner. Thus, this could represent a form of intrinsic bet-hedging and a source for added variation in the egg bank (Furness et al., 2015). Furness suggests that evolutionary bet-hedging guarantees that a portion of the embryos will develop successfully for a given set of actual conditions. The stress tolerance of *A. limnaeus* peaks at DII and decreases with post-DII development, as seen with anoxia, salinity, oxidative stress, and now dehydration stress (Machado and Podrabsky, 2007; Podrabsky et al., 2007; Riggs and Podrabsky, 2017; Wagner et al., 2019). By slowing post-DII development during aerial exposure, embryos maintain greater stress tolerance, which may be crucial for survival until favorable conditions return.

It is likely that *A. limnaeus* populations survive the dry seasons through entrance into DII (Podrabsky et al., 2016; Podrabsky et al., 2001). A survey of African annual killifish embryos of the genus *Nothobranchius* in the field showed

an egg bank primarily of DII embryos during the peak of dry season (Polačik et al., 2020). This is consistent with the remarkable tolerance of *A. limnaeus* embryos in DII to aerial conditions. However, despite post-DII embryos having less tolerance to dehydration stress than DII embryos, all stages measured survived over 100 d in aerial conditions, which may be long enough to survive a typical dry season if soil conditions remain at RH values of 85% or greater. That being said, much remains unknown about development under aerial conditions. Though not observed in these experiments, and not observed in the wild, DII embryos have been seen to break DII during aerial incubation (J.E.P., personal observations). The rate at which embryos develop after breaking DII under aerial incubation is unknown and is worth exploring. Additionally, exploration into the developmental rate of aerially incubated post-DII embryos when oxygen limitation is not a factor is of interest.

In summary, we show for the first time that embryos of *A. limnaeus* can survive aerial incubation for well over a year. Our findings are consistent with expected life history traits and known stress tolerance across development in *A. limnaeus*. Comparing aerial and aqueous oxygen consumption rates yielded unexpected outcomes and provide supporting evidence for the unique physiology of the annual killifish embryos in response to dehydration stress when compared to other fishes. Much remains unknown about survival of *A. limnaeus* embryos during aerial incubation. Further exploration at the metabolic level may provide insight into how this species survives in a dry substrate where they spend the majority of their lifecycle.

Table 3.1. Oxygen consumption (VO₂) of *Austrofundulus limnaeus* embryos incubated in aerial or aqueous conditions

Replicate	Parameter	Diapause II		Post-diapause II			
		Aerial	Aqueous	Aerial		Aqueous	
		Overall	Overall	WS 36	WS 38-42	WS 36	WS 38-42
1	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)	0.495	0.148	0.69	6.36	1.11	n /a
	R ²	0.94	0.99	0.86	0.97	0.95	
	P-value	< 0.0001	< 0.0001	0.0075	0.0017	< 0.0001	
2	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)	0.582	0.170	1.82	6.11	1.13	n /a
	R ²	0.92	0.97	0.98	1.00	0.96	
	P-value	< 0.0001	< 0.0001	0.0001	0.0001	< 0.0001	
3	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)	0.610	0.164	0.34	5.58	1.20	n /a
	R ²	0.99	0.96	0.99	0.99	0.98	
	P-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	
4	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)	0.823	0.120	0.66	4.69	1.33	n /a
	R ²	0.99	0.98	0.88	0.99	0.97	
	P-value	< 0.0001	< 0.0001	0.0005	0.0005	< 0.0001	
5	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)			1.71	3.58	1.11	n /a
	R ²			0.92	0.99	0.94	
	P-value			0.0025	0.0004	< 0.0001	
6	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)			1.046	1.12		
	R ²			0.94	0.95		
	P-value			0.0012	0.0048		
7	VO ₂ (nmol O ₂ h ⁻¹ embryo ⁻¹)			2.05	5.95		
	R ²			0.98	0.99		
	P-value			0.0002	0.0006

Figure 3.1. Survival of *Austrofundulus limnaeus* embryos incubated in aerial conditions. (A) Embryos were exposed to aerial conditions (85% RH) at 25°C without light in groups of 20 starting at the developmental stage listed. Post-diapause II embryos continue to develop under aerial incubation conditions. WS, Wourms's stage; DII, diapause II. Symbols are means $\pm$ SEM ($N = 3$). (B) Lethal time to 50% mortality (LT₅₀) for embryos exposed to aerial conditions. Different letters indicate significant differences in LT₅₀ between developmental stages (one-way ANOVA with Tukey's HSD test, $P < 0.05$). Bars are means $\pm$ SEM ($N = 3$) and symbols represent individual replicates.

Figure 3.2. Oxygen consumption of *Austrofundulus limnaeus* embryos in aerial and aqueous conditions. (A,B) Percentage of initial oxygen in the closed system respirometry vials over time. Note: total initial oxygen content in aerial vials was 33 times more than that of aqueous vials. Symbols are means $\pm$ SEM ($N = 4-7$) and each replicate contained 5 embryos per vial. (C,D) The amount of oxygen (μmol) consumed over time by embryos in aerial and aqueous treatments. (E,F) The amount of oxygen (μmol) in each aerial exposure vial over time. Inset graphs are for aqueous conditions. Rates of oxygen consumption were based on the slope of oxygen consumed over time. For DII embryos, a single slope was used for the entire time-course. For post-diapause II embryos, an initial slope and a final slope were calculated. See Table 3.1 for results of regression analyses.

Figure 3.3. Rate of oxygen consumption in *Austrofundulus limnaeus* embryos exposed to aqueous and aerial conditions. (A) Diapause II embryos exposed to aerial conditions had a significantly higher rate of oxygen consumption than those exposed to aqueous conditions (unpaired t-test, $P = 0.0005$). Bars are means $\pm$ SEM ($N = 4$) and symbols represent individual replicates. (B) Wourms' stage 36 embryos (initial rate) exposed to aerial conditions had a mean rate of oxygen consumption similar to those incubated in aqueous conditions, although the variance is clearly higher in aerial conditions (one-way ANOVA with Tukey's HSD, $P = 0.9999$). Final rates of oxygen consumption under aerial conditions in post-diapause II embryos varied considerably by replicate due to differences in developmental rates in embryos within each replicate as indicated by (C) the developmental stage of embryos in each vial at the end of the experiment. Different letters indicate significant differences in rates of oxygen consumption (one-way ANOVA with Tukey's HSD test, $P < 0.01$). For final measurements, symbols in B and C correspond to those used in Figure 3.2F.

Chapter 4

Metabolomics analysis provides insight on survival of annual killifish (*Austrofundulus limnaeus*) embryos during dehydration stress

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Introduction

The annual killifish, *Austrofundulus limnaeus*, inhabits temporary mud puddles in the Maracaibo basin of Venezuela. Their habitat is defined by relatively predictable patterns of average seasonal precipitation, but highly unpredictable temporal and spatial patterns of episodic rain events. This pattern of water availability leads to a great deal of uncertainty in the length of time that a puddle will experience inundation. Thus, individual puddles may remain dry for months or perhaps years (Podrabsky et al., 2016; Podrabsky et al., 1998; Polačik and Podrabsky, 2015). In fact, individuals may spend the vast majority of life span as embryos encased in dry mud. During the rainy season, adult *A. limnaeus* spawn and deposit their embryos into the muddy and often oxygen-limited sediment. As the rainy season comes to an end, the adult fish die and embryos must survive severely dehydrating conditions, often faced with a variety of other stresses such as extremes in temperature and oxygen until the wet season

returns and they can complete development (Podrabsky et al., 2016). As a result, *A. limnaeus* has evolved extremely stress-resistant embryos. Survival of these embryos is attributed, in part, to their ability to enter a profound state of metabolic depression termed diapause as part of their normal embryonic development (Podrabsky and Hand, 1999; Podrabsky and Hand, 2000). Entry into diapause allows for cessation of development, thus extending embryological survival during the dry season. There are three unique stages of diapause (I, II, and III) which an embryo can enter during development (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c). The most stress-tolerant stage, diapause II (DII), occurs midway through development and at this time the embryo mostly consists of cardiac and neural tissue (Podrabsky and Hand, 1999; Podrabsky et al., 2007). The developmental distribution of *A. limnaeus* embryos has not been characterized in the field. However, embryos of other species of annual killifish are primarily found in DII during the peak of the dry season, and in a variety of post-DII stages during the late dry season. Thus DII is likely primarily responsible for dry season survival in *A. limnaeus*, but dehydration tolerance is required during the entire duration of post-DII development. Even when embryos break DII and continue developing, they exhibit remarkable tolerance to stress that decreases as they progress towards hatching (Podrabsky et al., 2016; Podrabsky et al., 2001; Polačik et al., 2020).

Organismal survival in the absence of water is in itself a feat, but even more so for aquatic animals that rely on water as their main source of gas and nitrogenous waste exchange. Embryos of *A. limnaeus* experience unique

resistance to desiccation that is not seen in other aquatic vertebrates (Podrabsky et al., 2001; Zajic et al., 2020). However, the desiccation tolerance of these embryos has received far less attention than other aspects of their biology. When embryos of *A. limnaeus* are exposed to dehydrating conditions, they initially respond by losing the water from their extraembryonic perivitelline space in the first few days before water loss is reduced and approaches near zero after a week (Podrabsky et al., 2001). Minimal water loss appears to occur within the embryonic compartment, and in fact the embryo appears to remain fully hydrated. Diapause II embryos of *A. limnaeus* can survive over 500 days of aerial exposure to 85% relative humidity (RH) without access to liquid water, while post-DII embryos can survive well over 100 days. Diapause II embryos respond to aerial incubation by increasing oxygen consumption. In contrast, post-diapause II (post-DII) embryos initially maintain similar levels of oxygen consumption and then experience a severe decrease in oxygen consumption later in development compared to their aqueous counterparts. The unique and unexpected responses to aerial incubation lead to questions about what is occurring at the molecular level to aid in survival. The remarkable survival of embryos during aerial incubation undoubtedly requires physiological responses to adapt to life without water that have long gone unexplored.

Although molecular oxygen is vital for organisms, it can have deleterious effects in excess (França et al., 2007). For aquatic organisms, aerial exposure represents a large increase in oxygen availability compared to most aquatic habitats. Thus, we may expect accumulation of metabolites during aerial

exposure to assist in combating reactive oxygen species (ROS). A main reason for desiccation injury is attributed to the increased formation of ROS and subsequent oxidative damage due to water stress (França et al., 2007). Free radical formation can lead to lipid peroxidation, denaturation of proteins, and DNA damage which ultimately can affect overall metabolism (Hansen et al., 2006). However, protection against such damage can partially be mitigated by accumulation of antioxidant metabolites, such as glutathione (GSH) (Masella et al., 2005). Embryos of *A. limnaeus* have a notable tolerance of oxidative stress (Wagner et al., 2019), but the role of antioxidants has not been investigated through the lens of dehydration stress.

To survive without water, embryos must reduce evaporative water loss; accumulation of compatible osmolytes, restructuring of membranes, or alteration of waste compounds may therefore be critical for survival. A common mechanism for survival during water stress involves accumulation of water substitutes (e.g., trehalose) that can stabilize biological structures by "replacing" water and maintain hydrogen bonds that are critical for maintenance of structure (Erkut et al., 2011). This mechanism allows for the organism to enter a state of anhydrobiosis and is exhibited in a number of species, including tardigrades (*Hypsibius dujardini*), brine shrimp cysts (*Artemia franciscana*), and the dauer larva stage of *Caenorhabditis elegans* (Crowe et al., 1984; Erkut and Kurzchalia, 2015; Erkut et al., 2011). Although embryos of *A. limnaeus* cannot survive complete loss of cellular water, the accumulation of organic osmolytes is likely to be important in supporting survival of long-term dehydration stress.

Austrofundulus limnaeus is a promising model organism for developmental physiology and ecology because of their tolerance of a plethora of stresses, annotated genome, and ability to alter developmental trajectories in the lab (Podrabsky et al., 2017; Podrabsky et al., 2010a; Romney et al., 2018; Wagner et al., 2018). The current study was performed to expand our knowledge of stress tolerance in *A. limnaeus* by elucidating the metabolic pathways that may be providing this species with its remarkable abilities to survive under extreme dehydration stress. We examine the metabolite profiles of dormant (DII) and actively developing (post-DII) embryos in response to short and long-term aerial incubation. Here we report abundant changes in the metabolite profiles of embryos exposed to dehydrating conditions. We show that actively developing and dormant embryos share metabolic pathways and accumulation of similar metabolites in response to dehydration. However, we also see developmentally distinct differences that suggest stage-specific responses to aerial incubation and dehydration stress. We show critical roles for amino acid and lipid metabolism that demonstrates embryos are not limited to carbohydrate reserves for survival. We identify known antioxidant and neuroprotective compounds, as well as unique metabolites that have not yet been seen in the dehydration literature. Our findings provide a novel view for understanding the long-term survival of aerially incubated embryos and provide a foundation for future functional studies.

Materials and Methods

Animal husbandry and embryo collection

Adult annual killifish were housed and embryos collected as previously described (Podrabsky, 1999). All work was performed under established protocols that were reviewed and sanctioned by the Portland State University Institutional Animal Care and Use Committee (PSU IACUC protocols #33 and 64). Briefly, adult fish were kept in male-female pairs and spawned semiweekly. Embryos were collected and stored at 25°C with no light in 15 x 100 mm plastic Petri dishes in media that resembles the environmental conditions from which adults were collected in 1995 (10 mmol l⁻¹ NaCl, 2.15 mmol l⁻¹ MgCl₂, 0.8 mmol l⁻¹ CaCl₂, 0.14 mmol l⁻¹ KCl, 1.3 mmol l⁻¹ MgSO₄) (Podrabsky, 1999; Podrabsky et al., 1998). For the first 4 days post-fertilization (dpf), embryo medium contained methylene blue (0.0001%) to prevent fungal infection. Embryos were then treated with two 5 min washes of a 0.01% sodium hypochlorite solution (separated by a 5 min rest in embryo medium) to prevent bacterial and fungal growth, as previously described (Podrabsky, 1999). Following sodium hypochlorite treatment, embryos were transferred to embryo medium containing 10 mg l⁻¹ gentamicin sulfate and allowed to develop to DII (32–64 d). To break DII, embryos were subjected to a temperature of 30°C and full spectrum light for 48 h (Meller et al., 2012). Following this treatment, embryos were sorted into synchronized cohorts of embryos by developmental stage (Wourms' stage [WS]) as described in Podrabsky et al. (2017).

Embryonic stages investigated

Experiments were performed on dormant (DII) and actively developing post-DII embryos (Podrabsky et al., 2017; Wourms, 1972b; Wourms, 1972c) to identify stage-specific responses to dehydration stress and to capture different levels of dehydration tolerance. DII embryos are metabolically dormant, have halted development, and have a lethal time to 50% mortality (LT_{50}) in aerial incubation (85% relative humidity [RH]) of 325 d (Zajic et al., 2020). DII embryos tend to stay in diapause during aerial exposure. Post-DII embryos are metabolically active and continue developing during aerial exposure when oxygen is not limiting. Dehydration tolerance is reduced during post-DII development. When exposed to aerial conditions during early post-DII development (WS 36, 4 days post-diapause II), dehydration tolerance is reduced to an LT_{50} of 84 d. Late post-DII embryos (WS 40–42) have a further reduced ability to survive aerial incubation and exhibit LT_{50} values around 28–29 d (Zajic et al., 2020).

Metabolomics analysis

Aerial incubation

Embryos were exposed to 85% relative humidity (RH) air at 25°C with no light in a sealed glass desiccator with a porcelain plate shelf (250 mm diameter, 08615B, Fisher Scientific, Hampton, NH, USA). Relative humidity was controlled by using a saturated solution of potassium chloride (750 ml) placed below the shelf and continually mixed with a stir bar to ensure uniform RH within the aerial portion of the chamber (Podrabsky et al., 2001; Winston and Bates, 1960). Prior to

exposure, embryos were treated with sodium hypochlorite (see above) and incubated in embryo medium containing 10 mg l⁻¹ gentamicin sulfate for 3 h prior to aerial exposure. Embryos were placed on filter pads containing 2.5 ml embryo medium containing gentamicin. Care was taken to make certain that single embryos were isolated and not touching other embryos. DII embryos were then exposed to 85% RH at 25°C without light and sampled at 0, 7, and 28 d. Post-DII embryos were first exposed at WS 36 (4 days post-diapause II) and were sampled at 0, 7, and 18 d. Post-DII embryos continued developing during aerial incubation, which is consistent with previous data (Zajic et al., 2020). At 7 d, embryos sampled were at WS 40 and by 18 d, embryos sampled were at WS 42/43. Six biological replicates ($N = 6$), comprised of 25 embryos each, were flash frozen with liquid N₂ and stored at -80°C until shipped to Metabolon for metabolite profiling. To better visualize the sampling regimen, see **Figure 4.1**.

Metabolon metabolomics analysis

Sample preparation and metabolomics analysis occurred at Metabolon, but is briefly detailed here. Samples were prepared using an automated MicroLab STAR system (Hamilton Company, Reno, NV, USA). Proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. This method ensured dissociation of small molecules bound to protein, trapped in the precipitated protein matrix, and recovery of chemically diverse metabolites. The resulting extract was divided into four fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS

methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, and one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI. To remove methanol, samples were placed briefly on a TurboVap (Zymark, Hopkinton, MA, USA). The sample extracts were stored overnight under nitrogen before preparation for analysis. Prior to analysis, sample extracts were reconstituted in solvents compatible to each of the four methods described above. Raw data was extracted, peak-identified, and quality control processed using Metabolon's proprietary hardware and software. At the time of analysis, identification of known biochemicals was based on comparison to metabolomic libraries of more than 3300 commercially available purified standard compounds. Several curation procedures were carried out to ensure high quality data and removal of those data representing system artifacts, mis-assignments, and background noise. The present dataset comprises a total of 673 compounds of known identity (metabolites). Data for each metabolite is presented relative to control samples ($t = 0$) and expressed as non-normalized, protein-normalized (Bradford assay), and DNA-normalized (Table 1). Due to the changing amount of water in the samples over the course of aerial incubation, and continued development in post-DII embryos, we have chosen to use the DNA-normalized data to most accurately reflect the relative amounts of metabolites per cell. Bradford protein concentration increases in a linear manner during dehydration while at the same time embryo mass decreases in a similar manner (Fig 4.2). We interpret this pattern as an artifact of

water loss (especially in the DII embryos which are dormant) and so normalization per protein would exaggerate fold changes.

Statistical analysis

Graphical and statistical analyses were performed using Prism 8.0 software (GraphPad, La Jolla, CA, USA) or through Metabolon's user interface. Statistical significance was set to $P < 0.05$ for all comparisons. For metabolomics data analysis, missing values due to being under the limit of detection of the instruments were imputed with the minimum value on a per metabolite basis. For each metabolite, raw counts were scaled to set the median across all samples for that metabolite to 1 and the data log transformed. Following normalization to Bradford protein or DNA concentration, Welch's two-sample t -test was used to identify metabolites that differed significantly between treatments, with a threshold for statistical significance set to $P < 0.05$. Additionally, an estimate of the false discovery rate (q -value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. A low q -value ($q < 0.10$) is an indication of high confidence in a result. Thus, only metabolites with $P < 0.05$ and $q < 0.10$ are considered significant in this study. Pathway enrichment analysis was performed using MetaboLync (Metabolon Inc.) with all metabolites and their pre-assigned pathways as background and reference pathways, respectively. Enriched pathways were calculated based on the following formula, where significance was defined as $P < 0.05$:

$$\text{Enrichment value} = (k/m)/((n-k)/(N-m))$$

Where: k, total number of significant metabolites in pathway; m, total number of detected metabolites in pathway; n, total number of significant metabolites; N, total number of detected metabolites in the study. A pathway enrichment value greater than one indicates that the pathway contains more significantly changed compounds relative to the study overall. Fisher's exact test ($P < 0.05$) was used to determine if pathway enrichment was significant.

Results and Discussion

This is the first study to look at the metabolomic profile of embryos of *A. limnaeus* exposed to aerial incubation conditions. We previously measured the tolerance of DII and post-DII embryos to aerial conditions and showed the remarkable ability for DII embryos to survive without access to liquid water for over 500 d (Zajic et al., 2020). The profound ability for embryos to survive over a year without water warranted an analysis of what is occurring at the metabolic level. It has been well-established that DII and post-DII embryos may respond to stress in a stage-specific manner (Podrabsky et al., 2012a; Riggs and Podrabsky, 2017). The metabolic profiles of DII and post-DII vary greatly, but due to continued development of post-DII embryos during dehydration, we cannot confirm if the metabolites accumulated in post-DII embryos are dehydration-specific or developmentally regulated. It is also important to note that the levels of metabolites reported in this paper are from whole embryos. The location of the metabolites, whether they be in the developing embryo, yolk, or perivitelline fluid,

is not known. Recognizing the limitations of this study, we introduce new insights into survival of embryos of *A. limnaeus* to aerial incubation by focusing on pathways and metabolites that change in a similar manner in both dormant (DII) and actively developing (post-DII) embryos. Importantly, continuous exposure to dehydrating conditions during post-DII development is likely the most ecologically relevant condition under which to study this species. Below we outline metabolic pathways and metabolites of interest that may be supporting the extreme tolerance of embryos of *A. limnaeus* to dehydration stress induced by aerial exposure.

Metabolomics profiles overview

We measured a total of 673 metabolites of known identity (Supplemental Table S4.1). A summary of the numbers of metabolites that achieved statistical significance ($P < 0.05$) using various methods of normalization are presented in Table 4.1. Here we focus on data normalized to total DNA content to take into account the developmental changes that occur in post-DII embryos. We provide lists of the top significantly downregulated and upregulated metabolites in response to short-term or long-term aerial incubation in DII and post-DII embryos (Tables 4.2–4.9). Although the method of normalization is important, it does not affect the statistical significance of the vast majority of compounds that experience large fold-changes during aerial incubation (see boldface metabolites in Tables 4.2–4.9). Overall metabolite abundance was found to generally increase following aerial exposures, with the exception of short-term incubation

of post-DII embryos. DII embryos had 204 metabolites that were significantly altered during short-term exposures, while long-term exposures led to significant changes in 267 metabolites. Generally, metabolites (> 85%) were upregulated under aerial conditions in DII embryos. Post-DII embryos had 553 metabolites that were altered during short-term exposures, of which 479 were downregulated. During long-term aerial incubation, 390 metabolites were altered, of which 212 were upregulated. These changes in metabolites, especially in dormant DII embryos, indicate a large-scale metabolic response to aerial conditions. The increase in the majority of measured metabolites in DII embryos suggests an active role in surviving dehydration stress. The increase in some metabolites is consistent with the observed rise in oxygen consumption seen in DII embryos when exposed to aerial conditions (Zajic et al., 2020). Although dormant and not developing, DII embryos appear to actively respond to dehydration stress induced by aerial incubation.

Superpathways

Metabolites were categorized as belonging to 1 of 8 superpathways (Fig 4.3). Of the metabolites measured, 70% belonged to lipid ($N = 306$) and amino acid ($N = 164$) metabolism. The remaining 30% belonged to nucleotide ($N = 70$), carbohydrate ($N = 42$), cofactors and vitamins ($N = 30$), xenobiotics ($N = 30$), peptide ($N = 20$), and energy metabolism ($N = 11$). Within each superpathway, metabolites were clustered based on 81 subpathways (Fig 4.3).

Enriched subpathways

Diapause II.

There were 7 subpathways, primarily from lipid and amino acid metabolism, that were enriched in DII embryos following short-term aerial incubation (Table 4.9). The most enriched pathways were associated with fatty acid metabolism (dihydroxy, dicarboxylate, and monohydroxy). There was a significant contribution of amino acid metabolism, specifically, tyrosine metabolism, methionine, cysteine, SAM, and taurine metabolism, and leucine, isoleucine, and valine metabolism. The only pathway outside of amino acid and lipid metabolism that was enriched was nicotinate and nicotinamide metabolism. In response to long-term aerial incubation, there were 4 enriched subpathways (2 amino acid and 2 lipid) found in DII embryos. The amino acid subpathways enriched are shared with those enriched during short-term exposure. Again, the most enriched pathway was fatty acid metabolism (acyl carnitine, long chain saturated). Another lipid metabolic pathway, monoacylglycerol metabolism, was also enriched.

Post-DII embryos.

In general, there was more variation in pathway enrichment in post-DII embryos compared to DII embryos (Table 4.9). There were 10 enriched subpathways in response to short-term ($N = 5$) and long-term ($N = 5$) aerial incubation. Most enriched pathways belonged to lipid and amino acid metabolism. There were three pathways that were consistently enriched during

short and long-term exposures: phosphatidylcholine (PC), gamma-glutamyl amino acid, and monoacylglycerol metabolism. Unique to short-term incubation was enrichment in urea cycle (arginine and proline) metabolism and diacylglycerol metabolism. Unique to long-term exposure was enrichment in aminosugar metabolism and purine (adenine containing) metabolism.

Shared metabolites (long-term dehydration)

Due to continued development of post-DII embryos during dehydration exposure, we chose to focus on metabolites that showed shared responses (upregulation or downregulation) in DII and post-DII embryos in response to aerial exposure. Since DII embryos remained in DII during aerial incubation, we can more easily attribute metabolic change as primarily a direct response to dehydration stress and not an effect of active development. Thus, metabolites that change in both stages are likely due to a metabolic response to aerial incubation. DII embryos have a suppressed metabolism, thus changes in metabolic profiles may not be apparent after short-term incubation. We therefore chose to focus on metabolites shared following long-term aerial incubation to adequately capture a metabolic profile of embryos in response to dehydration stress.

When compared to their respective hydrated control, there were a total of 71 shared metabolites that significantly increased during long-term aerial incubation (Fig 4.4); whereas there were only 18 metabolites that significantly decreased during long-term exposures (Fig 4.4). Of the metabolites that

increased, 48 belonged to lipid ($N = 24$) and amino acid ($N = 24$) metabolism. The remainder belonged to carbohydrate ($N = 8$), nucleotide ($N = 7$), xenobiotics ($N = 6$), cofactors and vitamins ($N = 1$), and peptide metabolism ($N = 1$). Of the metabolites that decreased, a majority belonged to lipid metabolism ($N = 11$), and all lipid metabolites were from the monoacylglycerol subpathway. The remaining downregulated metabolites belonged to nucleotide ($N = 2$), cofactors and vitamins ($N = 2$), amino acid ($N = 1$), carbohydrate ($N = 1$), and xenobiotics ($N = 1$) metabolism.

Many of these metabolites are in pathways found to be enriched in response to short or long-term aerial incubation (Table 4.9). In addition to those enriched pathways, there are several metabolites that belong to shared pathways that will be explored further. Below we introduce metabolites, sorted by superpathway and subpathway, that were significantly upregulated or downregulated in embryos in response to long-term aerial exposure that we will explore further.

Pathways of interest

Amino acids and dipeptides

Methionine, Cysteine, and Gamma-Glutamyl Amino Acids. There are indications of altered transsulfuration activity and antioxidant demand and utilization in DII and post-DII embryos exposed to aerial incubation (Fig 4.5). The transsulfuration pathway is a highly conserved pathway that is involved in metabolizing sulfur-containing amino acids such as methionine and cysteine.

Methionine, via the transsulfuration pathway, can be converted to cysteine and several other compounds that play roles in redox balance and antioxidant defense (e.g., glutathione, taurine, etc.). Notably, an increase in the transsulfuration intermediate cystathionine is observed in both DII and post-DII embryos (Figs 4.4, 4.5).

The metabolite with the largest fold-change increase in response to aerial incubation is lanthionine (Fig 4.4; greater than 800-fold increase in post-DII embryos). Lanthionine is formed when cysteine is metabolized in lieu of homocysteine or cystathionine by the transsulfuration pathway enzymes cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) (Fig 4.5). A common product of the transsulfuration pathway, and specifically for lanthionine biosynthesis, is hydrogen sulfide (H_2S), which has been recognized as an important biological signaling molecule (Chiku et al., 2009; Elrod et al., 2007; Olson, 2018). H_2S has been shown to protect heart mitochondrial function during ischemic events and induce a state of suspended animation associated with a lower metabolism and body temperature in mice (Blackstone et al., 2005; Elrod et al., 2007). In addition, lanthionine can also be further metabolized in mammalian brain tissue to form the cyclic thioether, lanthionine ketimine, which is known to be associated with high affinity (58 nmol) to neuronal membranes (Fontana et al., 1990; Ricci et al., 1989) and has been demonstrated to have neuroprotective, neurotrophic and anti-inflammatory activities in mice during cerebral ischemia (Hensley et al., 2010; Nada et al., 2012). Embryos of *A. limnaeus* may experience dehydrating conditions for months and perhaps years

before water returns (Podrabsky et al., 2016; Polačik and Podrabsky, 2015). Long-term survival of dehydrating conditions very likely depends on suppression of metabolism and induction of cellular protective mechanisms, either through entrance into diapause II, or through induction of a dehydration-induced quiescence (Zajic et al., 2020). Thus, it is possible that lanthionine accumulation may support long-term survival of dehydration stress through suppression of metabolism mediated by H₂S production, and through the cytoprotective actions of lanthionine ketimine in cardiac and neural tissues.

The large increase in the relative abundance of lanthionine suggests a potential role as a compatible osmolyte during conditions of water limitation in embryos of *A. limnaeus*. However, in the relatively few studies that exist, elevated lanthionine appears to be a toxin and to have generally negative effects on fish embryo physiology and development. When zebrafish (*Danio rerio*) embryos were treated with lanthionine (0.3–2 µmol l⁻¹), abnormal development in heart chambers as well as increased heart rate and arrhythmia were observed, though some of these effects were counteracted with addition of glutathione (Perna et al., 2018). The potential toxic effects were further bolstered by evidence of lanthionine interfering with angiogenic signaling, increasing intracellular calcium, and impairing H₂S production (Perna et al., 2018; Vigorito et al., 2019). Thus, the ability of *A. limnaeus* embryos to tolerate such large increases in lanthionine without any apparent negative effects is an interesting result. Although we do not know the concentrations of lanthionine in embryos of *A. limnaeus*, an 800-fold change from even the picomolar level would reach

biologically relevant concentrations as reported for *D. rerio* embryos. The biology of lanthionine, and its potential association with the production of H₂S warrants further investigation into its role during development and in response to dehydration stress in embryos of *A. limnaeus*.

Both the oxidized and reduced forms of glutathione (GSSG and GSH, respectively) were found to be accumulated in post-DII embryos, whereas only GSSG was increased in abundance in DII embryos. Cells use glutathione to scavenge reactive oxygen species (ROS) in order to reduce oxidative damage. A previous study showed a significant increase in total glutathione content in *A. limnaeus* embryos during post-DII development (Wagner et al., 2019). However, close to 100% of the glutathione measured was in its reduced form, thus the increases in GSSG observed in this study appear to be the result of aerial incubation. In their natural habitat, *A. limnaeus* embryos are exposed to a variety of conditions (e.g. hypoxia, high temperatures) as they endure the desiccating conditions of the dry season, which could lead to increases in ROS production. A rise in GSSG is usually indicative of some form of oxidative stress. One explanation for the observed rise in GSSG in DII embryos may be due to increased oxidative stress as a consequence of increased oxygen availability and increased oxygen consumption observed in aerially incubated DII embryos (Zajic et al., 2020). Another possibility is that GSSG is assisting in reducing protein synthesis during dehydration, as GSSG is a known potent inhibitor of protein synthesis (Ernst et al., 1978). In response to dehydration, the star moss (*Tortula ruralis*) shows a similar rise in GSSG and subsequent decrease in protein

synthesis. It is not until rehydration that GSSG is rapidly converted to GSH and protein synthesis resumes.

Post-DII embryos also display significant decreases in 5-oxoproline, an intermediate of gamma-glutamyl amino acid metabolism, which could be reflective of changes in glutathione recycling. Changes in metabolites associated with the gamma-glutamyl amino acid subpathway were enriched during short- and long-term dehydration stress in post-DII embryos (Table 4.9). Fourteen gamma-glutamyl metabolites decreased in aerially incubated post-DII embryos (Fig 4.6). Only one metabolite, gamma-glutamyltryptophan, increased in both DII and post-DII embryos following long-term dehydration, while 4 additional gamma-glutamyl amino acids increased in DII embryos. There has been a lot of controversy in regards to the function of the gamma-glutamyl cycle in the literature. The original theory suggested the main function was transportation of amino acids into the cell via their conversion to gamma-glutamyl amino acids, a glutathione-dependent process (Orlowski and Meister, 1970). The release of amino acids from gamma-glutamyl amino acids by gamma-glutamyl cyclotransferase generates 5-oxoproline as an intermediate (Fig 4.5). However, recent findings suggest a role of the gamma-glutamyl cycle, not simply as an amino acid transporter, but as a regulator of redox metabolism of free radicals and xenobiotics (Inoue, 2016). A recent review by Bachhawat and Yadav proposes a “glutathione cycle” to replace the established gamma-glutamyl cycle (Bachhawat and Yadav, 2018). The discrepancies in these cycles is beyond the scope of this article, but there is a clear role for both glutathione and gamma-

glutamyl metabolism in *A. limnaeus* embryos worth further exploration. The changes in these metabolites is consistent with potential alterations in transsulfuration activity and antioxidant mobilization in response to aerial incubation and dehydration stress.

Tryptophan Metabolism. There were 2 metabolites that increased in the pathway for metabolism of tryptophan: C-glycosyltryptophan and picolinate. Of note is picolinate, which exhibited a profound fold-change (65-fold) in DII embryos and also a sequential increase in both stages during short- and long-term dehydration (Fig 4.4; Tables 4.2–4.5). The large fold-change increase observed in developmentally dormant embryos suggests an important role of picolinate. Picolinate has been found to have a wide range of neuroprotective, immunological, and anti-proliferative effects (Bosco et al., 2000; Fernandez-Pol et al., 1977; Grant et al., 2009; Ruffmann et al., 1987). Further investigations into a role for picolinate as a possible stress-induced cytoprotective molecule are a necessary next step in evaluating its potential importance.

Creatine Metabolism. DII and post-DII embryos exhibited different creatine metabolite profiles. Of note is the increase of creatine phosphate in DII embryos, and the sequential decrease of creatine phosphate in post-DII embryos in response to aerial exposure and dehydration stress (Fig 4.7). The reversible reaction of creatine to creatine-phosphate via creatine kinase generates diffusible high-energy phosphate reserves in the form of creatine phosphate. The

accumulation of creatine phosphate in DII embryos indicates a positive energy balance and is consistent with previous reports of high ATP/ADP ratios and high adenylate energy charge values during diapause (Podrabsky and Hand, 1999). The decline in creatine phosphate and subsequent rise in creatine in post-DII embryos suggests that ATP demand is outpacing ATP production leading to the depletion of the creatine phosphate pool. This may suggest that aerial exposure is a metabolic stress for post-DII embryos and not for DII embryos. In addition to its role in buffering cellular ATP levels, creatine and creatine phosphate have been shown to exhibit a number of cytoprotective effects including membrane stabilization (Tokarska-Schlattner et al., 2012). Creatine phosphate can directly bind to and stabilize liposome membrane phospholipids by altering phase transition temperatures and reducing membrane leak (Tokarska-Schlattner et al., 2012). Additionally, it has been shown that phosphocreatine can have protective effects against ischemic injury (Landoni et al., 2016; Saks and Strumia, 1993). Thus, the accumulation of creatine phosphate in DII embryos may offer protective effects that contribute to their much higher tolerance of aerial exposure and dehydration stress compared to post-DII embryos.

Glutamate Metabolism. The only metabolite in the glutamate subpathway to be upregulated in both DII and post-DII embryos was gamma-aminobutyrate (GABA) (Fig 4.4; Table 4.5). GABA is known to accumulate during exposure to anoxia in embryos of *A. limnaeus* (Podrabsky et al., 2007; Zajic and Podrabsky, 2020). DII embryos exhibited a 1.6-fold increase in GABA while post-DII embryos

showed a 20-fold increase following long-term incubation. When GABA is measured spectrophotometrically, it is often undetectable during normal *A. limnaeus* development (Zajic and Podrabsky, 2020). Thus, the amount of GABA accumulating during dehydration stress probably represents micromolar quantities, and not millimolar levels as seen during anoxia (Zajic and Podrabsky, 2020). GABA, in the micromolar level, is known to have neuroprotective roles in organisms (Hylland and Nilsson, 1999; Nilsson et al., 1991). GABA has also been shown to be important in reducing ROS production and protecting against oxidative stress in yeast (Cao et al., 2013; Coleman et al., 2001). Although the levels are likely lower than during anoxia, GABA may still have an important role as a neuroprotective and antioxidant agent during exposures to dehydration.

Carbohydrate

Glycolysis, Gluconeogenesis, and Pyruvate Metabolism and the Pentose Phosphate Pathways. There were 4 metabolites from these pathways that increased in both DII and post-DII embryos following long-term dehydration: Glucose-6-phosphate, pyruvate, lactate, and sedoheptulose-7-phosphate (Figs 3,7). There were also two stage-specific metabolites that accumulated: DII embryos accumulated glycerate, whereas post-DII embryos accumulated phosphoenolpyruvate (PEP) (Fig 4.8; Tables 4.2–4.4). We previously hypothesized that aerial incubation would limit gas exchange with the environment in concert with the observed decrease in evaporative water loss (Podrabsky et al., 2001) and lead to self-imposed hypoxia or anoxia. Although we

did observe results supporting this hypothesis in post-DII embryos, DII embryos respond in an opposite manner by increasing oxygen consumption (Zajic et al., 2020). This previous result is supported by the relatively small accumulation of lactate observed in DII embryos compared to post-DII embryos in response to dehydration stress. Thus, these data are consistent with hypoxic metabolism in post-DII but not DII embryos during long-term dehydration stress. Interestingly, the increase in lactate resembles that of the Warburg effect, which is commonly observed in many cancer cells, where enhanced glycolysis and lactate fermentation occur even in the presence of sufficient oxygen. Relying partially on anaerobic mechanisms may offset the reliance on aerobic metabolism which can ultimately lead to increases in ROS. Additionally, lactate in appropriate amounts has been shown to help regulate gene expression through inhibition of histone deacetylase activity and post-translational modifications of histones, act in a neuroprotective role by preventing excitotoxic cell death, and promote oxidative stress resistance (Berthet et al., 2009; Jourdain et al., 2016; Latham et al., 2012; Tauffenberger et al., 2019; Zhang et al., 2019). Thus, aerobic accumulation of lactate may help fuel a response to dehydration stress through a variety of mechanisms outside of its role in the maintenance of cytoplasmic redox balance.

The accumulation of glucose-6-phosphate and sedoheptulose-7-phosphate (S7P) is consistent with activation of the pentose phosphate pathway (PPP; Fig 4.8) (Patra and Hay, 2014). Shunting of carbon into the PPP results in the production of NADPH used to support anabolic processes, and also critical for the regeneration of reduced glutathione (Minard and McAlister-Henn, 2001;

Patra and Hay, 2014). Accumulation of S7P, but not glyceraldehyde-3-phosphate (G3P) suggests that the oxidative portion of the PPP is used to produce NADPH, but not all of the carbon is shuttled back into glycolysis and instead is allowed to accumulate as S7P. In addition, the accumulation of several ribose metabolism intermediates (Fig 4.4) is also consistent with increased flux through the PPP. This hypothesis provides a mechanism to support antioxidant capacity (see above discussion of glutathione) while allowing limited immediate carbon flow through glycolysis in the form of G3P, and accumulating a source of carbon (S7P) that could be readily utilized by glycolysis at a later time. These data suggest a critical role for the PPP in supporting aerial incubation and dehydration stress in *A. limnaeus* embryos.

Lipids

Fatty Acid, Dicarboxylate. There were 7 metabolites that increased following long-term dehydration: Glutarate (C5-DC), 3-methylglutarate/2-methylglutarate, 2-hydroxyglutarate, adipate (C6-DC), 3-hydroxyadipate, maleate, and sebacate (C10-DC) (Fig 4.4). Of interest is the accumulation of known oncometabolite 2-hydroxyglutarate (2-HG) (Sulkowski et al., 2020; Ward et al., 2010). There are two enantiomers of 2-HG, (S)2-HG and (R)2-HG, each of which can inhibit alpha-ketoglutarate-dependent enzymes involved in many biological processes (Xu et al., 2011). The methodology used to measure 2-HG in this study cannot discern enantiomers, thus further exploration needs to be done to see which form of the metabolite is found in embryos. Mutations in

isocitrate dehydrogenase associated with cancers produce (R)2-HG, which can stimulate cancer cell proliferation and inhibit TET enzymes and histone demethylases (Charitou et al., 2015; Intlekofer et al., 2017). In contrast, (S)2-HG is produced during oxygen limitation to regulate histone methylation levels and to help mitigate cellular reductive stress through inhibition of glycolysis and electron transport in mammalian cells (Intlekofer et al., 2015; Oldham et al., 2015). Formation of (S)2-HG can be formed through lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) via 'promiscuous' reduction of the alternative substrate alpha-ketoglutarate (Intlekofer et al., 2015; Intlekofer et al., 2017). Independent of enantiomer-specificity, 2-HG has been shown to inhibit ATP synthase and extend lifespan of *Caenorhabditis elegans* (Fu et al., 2015). 2-HG can have a number of effects on *A. limnaeus* embryos that could be beneficial in supporting survival during aerial incubation and dehydration stress.

Fatty Acid, Acyl carnitine. Several acyl carnitine metabolites ($N = 5$) were upregulated: arachidoylcarnitine (C20), arachidonoylcarnitine (C20:4), docosapentaenoylcarnitine (C22:5n6), stearoylcarnitine (C18), palmitoleoylcarnitine (C16:1) (Fig 4.4). Acyl carnitines play an essential role in regulating the balance of intracellular sugar and lipid metabolism (Li et al., 2019). Acyl carnitines primarily serve a functional role as carriers to transport long-chain fatty acids into mitochondria for β -oxidation, which is a major source of energy for cellular activities (Li et al., 2019; Tarasenko et al., 2018). Accumulation of these

metabolites may suggest reduced mitochondrial fatty acid metabolism during aerial incubation.

Phospholipids. The only metabolite in the phospholipid subpathway to be upregulated in both DII and post-DII embryos was cytidine 5'-diphosphocholine (Fig 4.4). Cytidine 5'-diphosphocholine is an intermediate product of phosphatidylcholine synthesis. The compound has been used extensively in clinical settings to treat patients with neurological disorders, including cerebral ischemia. The main protective role of cytidine 5'-diphosphocholine is through membrane stabilization (Zweifler, 2002). Further research has shown cytidine 5'-diphosphocholine protects rat livers from ischemia and reperfusion injury, thus preserving mitochondrial function and reducing oxidative stress (Zazueta et al., 2018). Reducing damage to oxygen-sensitive tissue, such as neural tissue, by stabilizing cell membranes and reducing free radical generation may be beneficial to survival of *A. limnaeus* embryos during aerial exposures and in response to dehydration stress.

Bile Acid Metabolism. Both tauroolithocholate and taurochenodeoxycholate accumulate during long-term aerial exposure. Tauroolithocholate accumulates to higher levels in DII embryos (almost 10-fold), while taurochenodeoxycholate exhibits an over 100-fold increase in post-DII embryos (Fig 4.4). In general, bile acids are toxic due to their ability to act as detergents, but the tauro-derivatives of bile acids tend to be less toxic (Rust et al., 2000). It is interesting that DII

embryos accumulate bile acids despite the absence of liver tissue. In addition to their role in digestion, bile acids can act as signaling molecules via a variety of pathways, including the regulation of a number of nuclear receptors (Hylemon et al., 2009). It is possible that these potentially toxic compounds are produced to activate signaling pathways that help support survival of aerial exposure. This possibility is intriguing and warrants further investigation.

Cofactors and Vitamins

Nicotinate and Nicotinamide metabolism. Metabolites linked to *de novo* and salvage (nicotinamide, nicotinamide riboside, nicotinamide ribonucleotide, and nicotinate) pathways of NAD⁺ synthesis were found to be significantly altered in response to aerial exposure in both DII and post-DII embryos. Of these, one metabolite was upregulated in both stages following long-term dehydration stress: nicotinamide (Fig 4.4). There were, however, 2 metabolites that were downregulated: nicotinate and nicotinamide ribonucleotide (NMN) (Fig 4.4). One difference between DII and post-DII embryos was the significant accumulation of nicotinamide adenine dinucleotide (NAD⁺) in DII embryos (Fig 4.9; 17-fold increase). NAD⁺ is a coenzyme that plays a vital role in redox reactions associated with glycolysis, the TCA cycle, and oxidative phosphorylation. Increasing NAD⁺ levels in mammals has been linked to improved mitochondrial function under stress (Canto et al., 2015; Cerutti et al., 2014; Khan et al., 2014). Enhancing NAD⁺ availability in *C. elegans* has been shown to increase lifespan and protect against ROS (Mouchiroud et al., 2013).

Conversely, reduction in NAD⁺ levels is an indication of senescence, as seen in various animal cell lines (Braidys et al., 2011; Canto et al., 2015; Gomes et al., 2013; Mouchiroud et al., 2013). Synthesis of NAD⁺ can occur via salvage and *de novo* pathways (Fig 4.9). *De novo* synthesis of NAD⁺ can occur via tryptophan metabolism by converting quinolinate to nicotinate ribonucleotide (NAMN). There is an initial rise in quinolinate in DII embryos followed by a decrease after long-term exposure. It is likely that the decrease in quinolinate contributed to the increase in NAD⁺ (Fig 4.9). The overall rise in NAD⁺ in DII embryos and maintained levels in post-DII embryos following long-term dehydration stress suggest changes in both demand and synthesis. The accumulation of NAD⁺ in DII suggests an available pool of NAD⁺ to contribute to metabolism, while the static levels of NAD⁺ in post-DII embryos suggests supply and demand are in synergy. The rise in the associated metabolites in the pathway provide a molecular supply for further synthesis of NAD⁺ via the salvage pathway.

Xenobiotics

Xenobiotics refer to compounds not expected to be present within an organism. There were several metabolites that were upregulated which are traditionally seen interacting with the microbiome. There were 3 metabolites upregulated in the tyrosine subpathway: phenol sulfate, 4-hydroxyphenylpyruvate, and 3-methoxytyrosine. Both DII and post-DII embryos exhibited an over 50-fold increase in phenol-sulfate following long-term dehydration (Fig 4.4). There were 3 metabolites in the benzoate pathway that

were upregulated following long-term dehydration: hippurate, guaiacol sulfate, p-cresol sulfate. Hippurate exhibited the largest fold change (over 100-fold) in DII embryos (Fig 4.4). Of these metabolites, hippurate and p-cresol sulfate are known markers for microbiome diversity (Lees et al., 2013; Pallister et al., 2017). Hippurate, along with other benzoates, has been shown to inhibit the growth of tumors in mouse cell lines (Spustova and Oravec, 1989). Much remains unknown about the role of benzoate metabolism outside of the microbiome and as a urinary waste product. The accumulation of these metabolites in *A. limnaeus* is interesting, as it is currently unknown whether fish embryos contain a maternally packaged microbiome that would explain these metabolites; this possibility is intriguing. While it is possible that these compounds accumulate due to microbial growth on the surface of the embryos, this is unlikely given the use of antibiotics in the medium, the detection of these metabolites at time zero, and the lack of other more general microbially-derived compounds that would be expected to accumulate. In addition, we observe the accumulation of bile acid metabolites in embryos that have not yet developed functioning liver tissue. Thus, there are many compounds that accumulate during dehydration stress that are inconsistent with our current knowledge of the metabolic capabilities of annual killifish embryos. There is evidence of specific enzymes in the yolk sacs of chicken and tortoise embryos that are capable of producing metabolites that otherwise only exist in microorganisms and plants (Chapeville and Fromageot, 1967). For example, chicken embryos are able to synthesize lanthionine from sulfate with the help of enzymes exclusively found in yolk sac endodermal cells. These

enzymes disappear upon hatching, which may help explain the lack of lanthionine in the literature. The possibility of these enzymes and metabolic capabilities in *A. limnaeus* is an exciting avenue worth further exploration. Alternatively, it is possible that these metabolites are produced by a yolk sac specific microbiome that aids in survival of dehydration stress. Further research needs to be done to elucidate the location, origin, and role of these metabolites in *A. limnaeus* embryos.

Conclusions and Future Directions

The goal of this metabolic profiling study was to gain biochemical insight into the mechanisms facilitating survival of aerial exposure and dehydration stress in *A. limnaeus* embryos. There is robust evidence of metabolic segregation in the study based on both embryonic stage and dehydration exposure, with changes following aerial exposure being significantly more pronounced in post-DII embryos, as expected. It also appears as though diapause II embryos, despite being metabolically dormant, are actively responding to dehydration tolerance by altering their metabolic profiles. There was an overwhelming downregulation trend seen in metabolites during short-term aerial exposure of post-DII embryos, which may indicate an initial response to elevated oxygen availability in the short-term and progressive imposition of hypoxia in long-term responses to aerial exposure and dehydration stress. However, further studies need to be done to delineate whether the responses seen in the post-DII embryos are developmental differences or dehydration-

specific responses. Despite this, there are an abundance of metabolites that change together in DII and post-DII embryos that may serve promising functions for supporting survival during aerial incubation and dehydration. In general, amino acid and lipid metabolism appear to play central roles in metabolic adjustments during aerial exposure, and it is clear that embryos are not solely relying on carbohydrate reserves to fuel metabolism. Increased capacity for detoxification of ROS and maintenance of redox balance appear to both be of major importance to supporting development under aerial conditions, and surviving dehydration stress. It is also interesting that several of the metabolites that experience large fold-change increases have previously been identified as neuroprotectants. This work offers a first glimpse into the metabolic programs that may support survival of aerial incubation and long-term survival of dehydration stress in embryos of *A. limnaeus* that can be functionally tested using genetic and pharmacological approaches.

Perspectives

Embryos of *A. limnaeus* likely spend many months, the preponderance of their life span, aeriually incubated in the wild. During aerial exposure, there are a number of metabolites accumulated that are usually associated with waste or have the potential to be toxic (e.g., bile acids, hippurate, lanthionine). Many of these compounds contain nitrogen or other typical waste products of metabolism that are excreted in the urine and feces. When exposed to aerial conditions, embryos of annual killifish must presumably rely on detoxification or

compartmentalization of waste product metabolites to ensure the developing embryo remains unharmed. One possible compartment for storage of waste products is the yolk. We previously suggested that lactate accumulated during anoxia may be buffered by transportation to the yolk, which is supported by the expression of several lactate transporter transcripts (Zajic and Podrabsky, 2020). It is possible that metabolites accumulated during aerial exposure are being shuttled into the yolk to protect the developing tissues, as seen with sequestration of ammonia in the yolk sac of rainbow trout (Steele et al., 2001). In amniotic eggs, the eggs of terrestrial vertebrates, waste is transported from the developing embryo into extraembryonic compartments, notably the amniotic, and the allantoic fluid (Aktuğ et al., 1998; Leeson and Leeson, 1963; Mellor and Slater, 1971; Ten Busch et al., 1997; Williams et al., 1993). In the anamniotic eggs of fish, waste products such as ammonia or bile acids can be relatively easily lost due to diffusion into the perivitelline fluid during early development, and excretion during late development as the chorion is not a substantial barrier for diffusion of relatively small metabolites (Peterson and Martin-Robichaud, 1987; Wright et al., 1995). However, in *A. limnaeus* the perivitelline fluid is quickly lost during aerial exposures and can no longer function as a mechanism for diffusive loss of metabolic waste products. Indeed, the embryos of annual killifish embryos exposed to aerial incubation have several unique features that approximate the functions of the shell and extraembryonic components of the amniotic eggs of terrestrial species. First, the chorion of annual killifish lacks pores and is substantially thicker than in most species of fish (Schoots et al.,

1982), and thus serves a protective function and as a partial barrier for larger molecules even under aquatic conditions (Schoots et al., 1982). Second, the enveloping cell layer of annual killifish embryos does not contribute to the skin of the developing embryos as it does in most fishes, instead it forms a syncytial membrane that surrounds the embryo and is shed upon hatching (Wourms, 1972a). This membrane constitutes the major permeability barrier between the embryo and its environment and has a unique structure with few embedded proteins (Jorgenson and Schmalbruch, 1984) and an extremely low permeability to water and salts (Machado and Podrabsky, 2007). The enveloping layer in annual killifishes effectively creates an extra-embryonic membrane-bound compartment that surrounds and protects the embryo in a manner similar to an amniotic chamber in terrestrial eggs. Thus, it is possible that a number of the compounds that are typically excreted as waste may be stored in either the yolk sac, or in extraembryonic fluid that is bound by the enveloping cell layer. In fact, many of the accumulated compounds identified in this study are known components of amniotic and allantoic fluid in other species (Aktuğ et al., 1998; Mellor and Slater, 1971; Ten Busch et al., 1997; Williams et al., 1993). Further exploration into the localization of these metabolites will help elucidate their possible function during aerial incubation and survival of dehydration stress.

Table 4.1. Total number of significant metabolites (among 673 total metabolites identified) that were significantly affected by short and long-term aerial exposure in DII and post-DII embryos initially exposed at WS 36.

Stage	Comparison	<u>Non-normalized</u>		<u>Protein-normalized</u>		<u>DNA-normalized</u>	
		Total Number <i>P</i> < 0.05	Number (up down)	Total Number <i>P</i> < 0.05	Number (up down)	Total Number <i>P</i> < 0.05	Number (up down)
DII	7 d / 0 d (short-term)	243	156 87	238	85 153	204	174 30
	28 d / 0 d (long-term)	346	298 48	200	77 123	267	233 34
Post- DII	7 d / 0 d (short-term)	466	383 83	438	269 169	553	74 479
	18 d / 0 d (long-term)	558	430 128	544	354 190	390	212 178

Table 4.2. Top 40 significantly upregulated metabolites during short-term aerial exposure in DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	phenol sulfate	39.52	Tyrosine Metabolism
2	tartarate	24.84	Food Component/Plant
3	alpha-hydroxycaproate	22.29	Fatty Acid, Monohydroxy
4	hippurate	20.11	Benzoate Metabolism
5	picolinate	16.79	Tryptophan Metabolism
6	nicotinamide adenine dinucleotide (NAD+)	16.34	Nicotinate and Nicotinamide Metabolism
7	p-cresol sulfate	14.51	Benzoate Metabolism
8	maleate	9.91	Fatty Acid, Dicarboxylate
9	3-indoxyl sulfate	9.83	Tryptophan Metabolism
10	1-carboxyethylphenylalanine	9.75	Phenylalanine Metabolism
11	hydroquinone sulfate	8.55	Drug - Topical Agents
12	guaiacol sulfate	8.36	Benzoate Metabolism
13	lanthionine	7.15	Methionine, Cysteine, SAM and Taurine Metabolism
14	salicylate	7.05	Drug - Topical Agents
15	sebacate (C10-DC)	6.97	Fatty Acid, Dicarboxylate
16	cysteine sulfinic acid	6.9	Methionine, Cysteine, SAM and Taurine Metabolism
17	quinolinate	6.74	Nicotinate and Nicotinamide Metabolism
18	tauro lithocholate	6.24	Secondary Bile Acid Metabolism
19	2-aminoheptanoate	5.89	Fatty Acid, Amino
20	arabinose	5.66	Pentose Metabolism
21	1-methyl-4-imidazoleacetate	4.81	Histidine Metabolism
22	benzoate	4.5	Benzoate Metabolism
23	glutarate (C5-DC)	4.37	Fatty Acid, Dicarboxylate
24	N-palmitoyltaurine	3.95	Endocannabinoid
25	undecanedioate (C11-DC)	3.63	Fatty Acid, Dicarboxylate
26	benzoylcarnitine*	3.52	Chemical
27	glycerate	3.36	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
28	glucuronate	3.34	Aminosugar Metabolism
29	ethylmalonate	3.33	Leucine, Isoleucine and Valine Metabolism
30	2-hydroxyheptanoate*	3.26	Fatty Acid, Monohydroxy
31	phytosphingosine	3.21	Sphingolipid Synthesis
32	1-methyl-5-imidazoleacetate	3.16	Histidine Metabolism
33	isovalerylcarnitine (C5)	3.07	Leucine, Isoleucine and Valine Metabolism
34	adipate (C6-DC)	3.02	Fatty Acid, Dicarboxylate
35	2-hydroxypalmitate	3	Fatty Acid, Monohydroxy
36	4-hydroxyphenylpyruvate	2.93	Tyrosine Metabolism
37	pyruvate	2.93	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
38	cysteine	2.92	Methionine, Cysteine, SAM and Taurine Metabolism
39	tartronate (hydroxymalonate)	2.92	Food Component/Plant
40	N-oleoyltaurine	2.88	Endocannabinoid

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.3. Top 40 significantly upregulated metabolites during long-term aerial exposure in DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	hippurate	101.49	Benzoate Metabolism
2	picolinate	64.9	Tryptophan Metabolism
3	phenol sulfate	53.01	Tyrosine Metabolism
4	tartarate	31.9	Food Component/Plant
5	maleate	20.29	Fatty Acid, Dicarboxylate
6	p-cresol sulfate	17.68	Benzoate Metabolism
7	nicotinamide adenine dinucleotide (NAD⁺)	16.82	Nicotinate and Nicotinamide Metabolism
8	arabinose	16.44	Pentose Metabolism
9	benzoylcarnitine*	15.64	Chemical
10	salicylate	14.33	Drug - Topical Agents
11	hydroquinone sulfate	13.42	Drug - Topical Agents
12	3-indoxyl sulfate	11.46	Tryptophan Metabolism
13	2-aminoheptanoate	10.16	Fatty Acid, Amino
14	guaiacol sulfate	9.77	Benzoate Metabolism
15	tauroolithocholate	8.67	Secondary Bile Acid Metabolism
16	glutarate (C5-DC)	8.47	Fatty Acid, Dicarboxylate
17	cysteine sulfinic acid	7.67	Methionine, Cysteine, SAM and Taurine Metabolism
18	glucuronate	4.98	Aminosugar Metabolism
19	1-methyl-4-imidazoleacetate	4.6	Histidine Metabolism
20	ethylmalonate	4.21	Leucine, Isoleucine and Valine Metabolism
21	adipate (C6-DC)	4.16	Fatty Acid, Dicarboxylate
22	cyano-alanine	4.14	Methionine, Cysteine, SAM and Taurine Metabolism
23	lanthionine	4.07	Methionine, Cysteine, SAM and Taurine Metabolism
24	sebacate (C10-DC)	3.89	Fatty Acid, Dicarboxylate
25	glycerate	3.83	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
26	methylsuccinate	3.8	Leucine, Isoleucine and Valine Metabolism
27	palmitoleoylcarnitine (C16:1)*	3.77	Fatty Acid Metabolism(Acyl Carnitine)
28	pheophorbide A	3.71	Food Component/Plant
29	phytosphingosine	3.59	Sphingolipid Synthesis
30	xylose	3.51	Pentose Metabolism
31	2-hydroxyglutarate	3.39	Fatty Acid, Dicarboxylate
32	3-methylglutarate/2-methylglutarate	3.34	Fatty Acid, Dicarboxylate
33	linoleoylcarnitine (C18:2)*	3.26	Fatty Acid Metabolism(Acyl Carnitine)
34	2'-deoxyinosine	3.18	Purine Metabolism, (Hypo)Xanthine/Inosine containing
35	thymol sulfate	3.18	Food Component/Plant
36	N-formylmethionine	3.08	Methionine, Cysteine, SAM and Taurine Metabolism
37	creatine phosphate	3.06	Creatine Metabolism
38	4-hydroxyphenylpyruvate	3	Tyrosine Metabolism
39	1-methyl-5-imidazoleacetate	2.97	Histidine Metabolism
40	glutathione, oxidized (GSSG)	2.95	Glutathione Metabolism

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.4. Top 40 significantly upregulated metabolites during short-term aerial exposure in post-DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	lanthionine	16.93	Methionine, Cysteine, SAM and Taurine Metabolism
2	N-acetyl-1-methylhistidine*	15.39	Histidine Metabolism
3	3-chlorotyrosine	8.24	Tyrosine Metabolism
4	phenol sulfate	8.15	Tyrosine Metabolism
5	pterin	8.05	Pterin Metabolism
6	N-acetyl-3-methylhistidine*	7.21	Histidine Metabolism
7	linolenoylcarnitine (C18:3)*	6.65	Fatty Acid Metabolism(Acyl Carnitine)
8	picolinate	5.1	Tryptophan Metabolism
9	allantoin	4.67	Purine Metabolism, (Hypo)Xanthine/Inosine containing
10	xanthopterin	4.03	Pterin Metabolism
11	taurochenodeoxycholate	3.74	Primary Bile Acid Metabolism
12	2'-O-methyluridine	3.56	Pyrimidine Metabolism, Uracil containing
13	kynurenate	3.31	Tryptophan Metabolism
14	guanine	3.16	Purine Metabolism, Guanine containing
15	pro-hydroxy-pro	3.09	Urea cycle; Arginine and Proline Metabolism
16	methionine sulfone	2.97	Methionine, Cysteine, SAM and Taurine Metabolism
17	arachidonoylcarnitine (C20:4)	2.94	Fatty Acid Metabolism(Acyl Carnitine)
18	N2-acetyllysine	2.8	Lysine Metabolism
19	mannose-6-phosphate	2.72	Fructose, Mannose and Galactose Metabolism
20	adenosine 3'-monophosphate (3'-AMP)	2.69	Purine Metabolism, Adenine containing
21	glucose 6-phosphate	2.68	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
22	methyl glucopyranoside (alpha + beta)	2.65	Food Component/Plant
23	2'-deoxyinosine	2.6	Purine Metabolism, (Hypo)Xanthine/Inosine containing
24	docosapentaenoylcarnitine (C22:5n6)*	2.6	Fatty Acid Metabolism(Acyl Carnitine)
25	uridine 2'-monophosphate (2'-UMP)*	2.56	Pyrimidine Metabolism, Uracil containing
26	palmitoleoylcarnitine (C16:1)*	2.36	Fatty Acid Metabolism(Acyl Carnitine)
27	glutathione, oxidized (GSSG)	2.24	Glutathione Metabolism
28	ceramide (d16:1/24:1, d18:1/22:1)*	2.12	Ceramides
29	uridine	2.12	Pyrimidine Metabolism, Uracil containing
30	betaine aldehyde	2.1	Glycine, Serine and Threonine Metabolism
31	myristoylcarnitine (C14)	2.1	Fatty Acid Metabolism(Acyl Carnitine)
32	linoleoylcarnitine (C18:2)*	2.1	Fatty Acid Metabolism(Acyl Carnitine)
33	sedoheptulose-7-phosphate	1.98	Pentose Phosphate Pathway
34	cystathionine	1.89	Methionine, Cysteine, SAM and Taurine Metabolism
35	alpha-ketobutyrate	1.64	Methionine, Cysteine, SAM and Taurine Metabolism
36	phosphoenolpyruvate (PEP)	1.64	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
37	cyano-alanine	1.62	Methionine, Cysteine, SAM and Taurine Metabolism
38	galactose 1-phosphate	1.62	Fructose, Mannose and Galactose Metabolism
39	4-acetamidobutanoate	1.59	Polyamine Metabolism
40	(3'-5')-adenylyluridine	1.58	Dinucleotide

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.5. Top 40 significantly upregulated metabolites during long-term aerial exposure in post-DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	lanthionine	868.97	Methionine, Cysteine, SAM and Taurine Metabolism
2	methyl glucopyranoside (alpha + beta)	207.74	Food Component/Plant
3	xanthopterin	133.64	Pterin Metabolism
4	taurochenodeoxycholate	132.19	Primary Bile Acid Metabolism
5	indoleacetylcarnitine*	98.21	Chemical
6	kynurenate	80.86	Tryptophan Metabolism
7	N-acetyl-1-methylhistidine*	75.31	Histidine Metabolism
8	adenosine 2'-monophosphate (2'-AMP)	68.14	Purine Metabolism, Adenine containing
9	phenol sulfate	58.17	Tyrosine Metabolism
10	biopterin	57.99	Tetrahydrobiopterin Metabolism
11	sedoheptulose	56.67	Pentose Metabolism
12	taurocholate	56.18	Primary Bile Acid Metabolism
13	N2-acetyllysine	52.59	Lysine Metabolism
14	dihydrobiopterin	50.04	Tetrahydrobiopterin Metabolism
15	N-acetyl-3-methylhistidine*	46.95	Histidine Metabolism
16	(N(1) + N(8))-acetylspermidine	41.13	Polyamine Metabolism
17	allantoin	39.12	Purine Metabolism, (Hypo)Xanthine/Inosine containing
18	cytidine 5'-diphosphocholine	35.47	Phospholipid Metabolism
19	7-methylguanine	33.21	Purine Metabolism, Guanine containing
20	glutathione, oxidized (GSSG)	31.19	Glutathione Metabolism
21	guanosine 2'-monophosphate (2'-GMP)*	29.95	Purine Metabolism, Guanine containing
22	3-chlorotyrosine	29.12	Tyrosine Metabolism
23	picolinate	28.53	Tryptophan Metabolism
24	alpha-ketobutyrate	27	Methionine, Cysteine, SAM and Taurine Metabolism
25	ribose	26.65	Pentose Metabolism
26	cystathionine	25.67	Methionine, Cysteine, SAM and Taurine Metabolism
27	isoxanthopterin	23.02	Pterin Metabolism
28	urate	21.73	Purine Metabolism, (Hypo)Xanthine/Inosine containing
29	hypoxanthine	21.39	Purine Metabolism, (Hypo)Xanthine/Inosine containing
30	gamma-aminobutyrate (GABA)	20.33	Glutamate Metabolism
31	uracil	18.77	Pyrimidine Metabolism, Uracil containing
32	uridine 2'-monophosphate (2'-UMP)*	18.57	Pyrimidine Metabolism, Uracil containing
33	N-acetylglucosamine 6-phosphate	18.18	Aminosugar Metabolism
34	argininate*	18.13	Urea cycle; Arginine and Proline Metabolism
35	ceramide (d16:1/24:1, d18:1/22:1)*	17.76	Ceramides
36	1-(1-enyl-palmitoyl)-2-linoleoyl-GPE (P-16:0/18:2)*	17.12	Plasmalogen
37	pterin	17.05	Pterin Metabolism
38	sedoheptulose-7-phosphate	16.82	Pentose Phosphate Pathway
39	adenosine 3'-monophosphate (3'-AMP)	16.29	Purine Metabolism, Adenine containing
40	betaine aldehyde	14.92	Glycine, Serine and Threonine Metabolism

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.6. Top 30 significantly downregulated metabolites during short-term aerial exposure in DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	nicotinate	0.01	Nicotinate and Nicotinamide Metabolism
2	ergothioneine	0.03	Food Component/Plant
3	hypotaurine	0.08	Methionine, Cysteine, SAM and Taurine Metabolism
4	N-acetylputrescine	0.16	Polyamine Metabolism
5	pheophytin A	0.18	Food Component/Plant
6	nicotinamide ribonucleotide (NMN)	0.19	Nicotinate and Nicotinamide Metabolism
7	1-docosaheptaenoylglycerol (22:6)	0.21	Monoacylglycerol
8	2-docosaheptaenoylglycerol (22:6)*	0.22	Monoacylglycerol
9	N-carbamoylaspartate	0.24	Pyrimidine Metabolism, Orotate containing
10	hexadecatrienoate (16:3n3)	0.27	Polyunsaturated Fatty Acid (n3 and n6)
11	1-myristoylglycerol (14:0)	0.28	Monoacylglycerol
12	1-palmitoleoylglycerol (16:1)*	0.28	Monoacylglycerol
13	tetradecadienoate (14:2)*	0.28	Polyunsaturated Fatty Acid (n3 and n6)
14	1-heptadecenoylglycerol (17:1)*	0.28	Monoacylglycerol
15	1-linoleoylglycerol (18:2)	0.29	Monoacylglycerol
16	1-dihomo-linolenylglycerol (20:3)	0.3	Monoacylglycerol
17	myristoleate (14:1n5)	0.31	Long Chain Fatty Acid
18	trans-uconate	0.34	Histidine Metabolism
19	2,3-dihydroxyisovalerate	0.36	Food Component/Plant
20	xanthine	0.39	Purine Metabolism, (Hypo)Xanthine/Inosine containing
21	4-hydroxy-nonenal-glutathione	0.41	Glutathione Metabolism
22	sucrose	0.46	Disaccharides and Oligosaccharides
23	hexadecadienoate (16:2n6)	0.47	Polyunsaturated Fatty Acid (n3 and n6)
24	adenosine	0.52	Purine Metabolism, Adenine containing
25	N-acetylmethionine	0.54	Methionine, Cysteine, SAM and Taurine Metabolism
26	adenine	0.54	Purine Metabolism, Adenine containing
27	guanine	0.55	Purine Metabolism, Guanine containing
28	dihomo-linoleoylcarnitine (C20:2)*	0.57	Fatty Acid Metabolism(Acyl Carnitine)
29	dihydroxyacetone phosphate (DHAP)	0.57	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
30	uracil	0.61	Pyrimidine Metabolism, Uracil containing

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.7. Top 34 significantly downregulated metabolites during long-term aerial exposure in DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	nicotinate	0.02	Nicotinate and Nicotinamide Metabolism
2	ergothioneine	0.06	Food Component/Plant
3	nicotinamide ribonucleotide (NMN)	0.15	Nicotinate and Nicotinamide Metabolism
4	N-acetylputrescine	0.16	Polyamine Metabolism
5	sucrose	0.16	Disaccharides and Oligosaccharides
6	1-heptadecenoylglycerol (17:1)*	0.17	Monoacylglycerol
7	1-docosaehaenoylglycerol (22:6)	0.21	Monoacylglycerol
8	guanine	0.22	Purine Metabolism, Guanine containing
9	tetradecadienoate (14:2)*	0.25	Polyunsaturated Fatty Acid (n3 and n6)
10	1-oleoylglycerol (18:1)	0.25	Monoacylglycerol
11	1-myristoylglycerol (14:0)	0.26	Monoacylglycerol
12	1-palmitoleoylglycerol (16:1)*	0.26	Monoacylglycerol
13	pheophytin A	0.27	Food Component/Plant
14	hexadecatrienoate (16:3n3)	0.27	Polyunsaturated Fatty Acid (n3 and n6)
15	1-dihomo-linolenylglycerol (20:3)	0.27	Monoacylglycerol
16	1-linoleoylglycerol (18:2)	0.28	Monoacylglycerol
17	2-heptadecenoylglycerol (17:1)*	0.29	Monoacylglycerol
18	myristoleate (14:1n5)	0.3	Long Chain Fatty Acid
19	2-docosaehaenoylglycerol (22:6)*	0.31	Monoacylglycerol
20	hypoxanthine	0.31	Purine Metabolism, (Hypo)Xanthine/Inosine containing
21	N-carbamoylaspartate	0.34	Pyrimidine Metabolism, Orotate containing
22	trans-urocanate	0.35	Histidine Metabolism
23	adenosine	0.36	Purine Metabolism, Adenine containing
24	hexadecadienoate (16:2n6)	0.37	Polyunsaturated Fatty Acid (n3 and n6)
25	4-hydroxy-nonanal-glutathione	0.41	Glutathione Metabolism
26	13-HODE + 9-HODE	0.44	Fatty Acid, Monohydroxy
27	3-hydroxybutyrate (BHBA)	0.45	Ketone Bodies
28	2-palmitoleoylglycerol (16:1)*	0.46	Monoacylglycerol
29	ophthalmate	0.48	Glutathione Metabolism
30	xanthine	0.52	Purine Metabolism, (Hypo)Xanthine/Inosine containing
31	adenine	0.53	Purine Metabolism, Adenine containing
32	dihydroxyacetone phosphate (DHAP)	0.57	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism
33	uracil	0.58	Pyrimidine Metabolism, Uracil containing
34	1-arachidonylglycerol (20:4)	0.61	Monoacylglycerol

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.8. Top 40 significantly downregulated metabolites during short-term aerial exposure in post-DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	nicotinate	0.01	Nicotinate and Nicotinamide Metabolism
2	adenosine 5'-monophosphate (AMP)	0.01	Purine Metabolism, Adenine containing
3	2'-deoxyguanosine 5'-monophosphate (dGMP)	0.02	Purine Metabolism, Guanine containing
4	5-oxoproline	0.03	Glutathione Metabolism
5	trans-urocanate	0.06	Histidine Metabolism
6	pipecolate	0.06	Lysine Metabolism
7	creatine phosphate	0.06	Creatine Metabolism
8	gamma-glutamylalanine	0.06	Gamma-glutamyl Amino Acid
9	gamma-glutamyl-2-aminobutyrate	0.07	Gamma-glutamyl Amino Acid
10	ergothioneine	0.08	Food Component/Plant
11	cystine	0.08	Methionine, Cysteine, SAM and Taurine Metabolism
12	sucrose	0.08	Disaccharides and Oligosaccharides
13	3-ureidopropionate	0.09	Pyrimidine Metabolism, Uracil containing
14	2'-deoxyadenosine 5'-monophosphate	0.09	Purine Metabolism, Adenine containing
15	N-acetylputrescine	0.1	Polyamine Metabolism
16	2-oxoarginine*	0.1	Urea cycle; Arginine and Proline Metabolism
17	hypotaurine	0.11	Methionine, Cysteine, SAM and Taurine Metabolism
18	N-acetylneuraminate	0.11	Aminosugar Metabolism
19	inosine 5'-monophosphate (IMP)	0.11	Purine Metabolism, (Hypo)Xanthine/Inosine containing
20	lysylleucine	0.12	Dipeptide
21	nicotinamide ribonucleotide (NMN)	0.12	Nicotinate and Nicotinamide Metabolism
22	thymidine 5'-monophosphate	0.13	Pyrimidine Metabolism, Thymine containing
23	4-imidazoleacetate	0.13	Histidine Metabolism
24	mannose	0.13	Fructose, Mannose and Galactose Metabolism
25	2'-deoxyadenosine	0.13	Purine Metabolism, Adenine containing
26	linoleoyl-arachidonoyl-glycerol (18:2/20:4) [1]*	0.14	Diacylglycerol
27	serine	0.14	Glycine, Serine and Threonine Metabolism
28	isocitrate	0.14	TCA Cycle
29	asparagine	0.15	Alanine and Aspartate Metabolism
30	aconitate [cis or trans]	0.15	TCA Cycle
31	N-carbamoylaspartate	0.16	Pyrimidine Metabolism, Orotate containing
32	isoleucylglycine	0.16	Dipeptide
33	1-palmitoleoylglycerol (16:1)*	0.16	Monoacylglycerol
34	2'-deoxyuridine	0.17	Pyrimidine Metabolism, Uracil containing
35	linoleoylcholine*	0.17	Fatty Acid Metabolism (Acyl Choline)
36	cysteine sulfinic acid	0.18	Methionine, Cysteine, SAM and Taurine Metabolism
37	alanine	0.18	Alanine and Aspartate Metabolism
38	2'-deoxycytidine	0.18	Pyrimidine Metabolism, Cytidine containing
39	glutamate	0.18	Glutamate Metabolism
40	pyridoxamine	0.18	Vitamin B6 Metabolism

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.9. Top 40 significantly downregulated metabolites during long-term aerial exposure in post-DII embryos normalized to DNA concentration.

Rank	Significant metabolite	Metabolite Fold change	Associated sub pathway
1	gamma-glutamyl-2-aminobutyrate	0.01	Gamma-glutamyl Amino Acid
2	inosine 5'-monophosphate (IMP)	0.01	Purine Metabolism, (Hypo)Xanthine/Inosine containing
3	gamma-glutamylglutamate	0.01	Gamma-glutamyl Amino Acid
4	5-oxoproline	0.03	Glutathione Metabolism
5	gamma-glutamylalanine	0.03	Gamma-glutamyl Amino Acid
6	adenosine 5'-monophosphate (AMP)	0.04	Purine Metabolism, Adenine containing
7	creatine phosphate	0.04	Creatine Metabolism
8	gamma-glutamylglycine	0.04	Gamma-glutamyl Amino Acid
9	isocitrate	0.05	TCA Cycle
10	gamma-glutamylserine	0.05	Gamma-glutamyl Amino Acid
11	oleoylcholine	0.06	Fatty Acid Metabolism (Acyl Choline)
12	uridine 5'-monophosphate (UMP)	0.06	Pyrimidine Metabolism, Uracil containing
13	sucrose	0.07	Disaccharides and Oligosaccharides
14	aconitate [cis or trans]	0.07	TCA Cycle
15	nicotinate	0.08	Nicotinate and Nicotinamide Metabolism
16	5,6-dihydrouridine	0.08	Pyrimidine Metabolism, Uracil containing
17	2'-deoxyadenosine	0.09	Purine Metabolism, Adenine containing
18	N-acetylneuraminate	0.1	Aminosugar Metabolism
19	maltotetraose	0.11	Glycogen Metabolism
20	pipecolate	0.12	Lysine Metabolism
21	2-palmitoleoylglycerol (16:1)*	0.12	Monoacylglycerol
22	guanidinoacetate	0.13	Creatine Metabolism
23	linoleoylcholine*	0.14	Fatty Acid Metabolism (Acyl Choline)
24	citrate	0.14	TCA Cycle
25	palmitoleoylcholine	0.15	Fatty Acid Metabolism (Acyl Choline)
26	2-arachidonoylglycerol (20:4)	0.15	Monoacylglycerol
27	palmitoylcholine	0.16	Fatty Acid Metabolism (Acyl Choline)
28	1-arachidonoylglycerol (20:4)	0.16	Monoacylglycerol
29	adenosine	0.16	Purine Metabolism, Adenine containing
30	stearoylcholine*	0.17	Fatty Acid Metabolism (Acyl Choline)
31	gamma-glutamylthreonine	0.17	Gamma-glutamyl Amino Acid
32	3-aminoisobutyrate	0.17	Pyrimidine Metabolism, Thymine containing
33	1-palmitoleoylglycerol (16:1)*	0.18	Monoacylglycerol
34	2'-deoxyguanosine 5'-monophosphate (dGMP)	0.19	Purine Metabolism, Guanine containing
35	maltopentaose	0.19	Glycogen Metabolism
36	N-acetylglucosamine/N-acetylgalactosamine	0.19	Aminosugar Metabolism
37	oleoyl-oleoyl-glycerol (18:1/18:1) [1]*	0.19	Diacylglycerol
38	4-hydroxyglutamate	0.2	Glutamate Metabolism
39	5-methyluridine (ribothymidine)	0.21	Pyrimidine Metabolism, Uracil containing
40	N-acetyl-aspartyl-glutamate (NAAG)	0.21	Glutamate Metabolism

Metabolites in bold are also found in the top 40 when normalized to protein content. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Table 4.10. Metabolic pathways that were significantly enriched by short and long-term aerial exposure in DII and post-DII embryos normalized to DNA concentration.

Treatment (# significant metabolites)	Subpathway	Enrichment Value	P-value	k	m
DII short-term (n = 204)	Fatty Acid, Dihydroxy	3.3	0.03	3	3
	Fatty Acid, Dicarboxylate	2.6	6.34E-04	10	13
	Fatty Acid, Monohydroxy	2.4	0.03	5	7
	Nicotinate and Nicotinamide Metabolism	2.4	0.03	5	7
	Tyrosine Metabolism	2.4	0.01	7	10
	Methionine, Cysteine, SAM and Taurine Metabolism	2.0	4.17E-03	13	22
	Leucine, Isoleucine and Valine Metabolism	1.9	0.02	11	20
DII long-term (n = 267)	Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	1.9	0.05	6	8
	Tyrosine Metabolism	1.8	0.05	7	10
	Monoacylglycerol	1.7	0.03	11	17
	Methionine, Cysteine, SAM and Taurine Metabolism	1.6	0.02	14	22
Post-DII short-term (n = 553)	Phosphatidylcholine (PC)	1.2	0.02	19	19
	Urea cycle; Arginine and Proline Metabolism	1.2	0.02	19	19
	Gamma-glutamyl Amino Acid	1.2	0.03	17	17
	Monoacylglycerol	1.2	0.03	17	17
	Diacylglycerol	1.2	7.71E-03	34	35
Post-DII long-term (n = 390)	Aminosugar Metabolism	1.7	0.01	8	8
	Purine Metabolism, Adenine containing	1.6	0.03	9	10
	Gamma-glutamyl Amino Acid	1.5	7.36E-03	15	17
	Monoacylglycerol	1.5	7.36E-03	15	17
	Phosphatidylcholine (PC)	1.5	0.01	16	19

Enrichment value was computed as follows: $(k/m)/((n-k)/(N-m))$, where: k, total number of significant metabolites in pathway; m, total number of detected metabolites in pathway; n, total number of significant metabolites; N, total number of detected metabolites (673). A pathway enrichment value greater than one indicates that the pathway contained more significantly changed metabolites relative to the study overall. Fisher's exact test was used to determine if pathway enrichment was significant ($P < 0.05$).

Figure 4.1. Schematic of the experimental design and sampling regimen used for metabolomics analysis. Embryos were exposed to 85% relative humidity (RH) at the developmental stage listed (DII or WS 36). Embryos were sampled after short-term (7 d) and long-term (18 or 28 d) aerial exposure ($N = 6$, each replicate contained 25 embryos). Developmental stage at time of sampling is included below each timepoint. DII embryos sampled remained in diapause, whereas WS 36 embryos continued developing. WS, Wourms' stage; DII, diapause II.

Figure 4.2. The relationship of aerial exposure on mass and protein concentration in DII and post-DII embryos. There is a 25% decrease in mass of embryos over the course of aerial incubation in DII and post-DII embryos. Bradford protein content increases by 34 and 38% over the course of aerial incubation in DII and post-DII embryos, respectively. Due to these systematic changes driven by water loss over the course of aerial exposure, normalization to protein exaggerates fold changes.

Figure 4.3. Heat maps of fold change from $t = 0$ of 673 metabolites detected in embryos. Heat maps (organized by superpathway) represent the relative $\log_2$ transformed fold change of each metabolite in response to short-term (S) and long-term (L) aerial incubation in diapause II (DII) and post-DII embryos when compared to hydrated control embryos at $t = 0$. Metabolites are clustered by subpathways. Major subpathways are indicated on the left of each heat map. Within each heatmap, fold changes are displayed for all comparisons, even though fold changes may not be statistically significant for all columns. For details on sampling, see Figure 4.1.

Figure 4.4. Metabolites significantly down or upregulated during long-term aerial exposure in DII and post-DII embryos. The $\log_2$ transformed fold changes of shared metabolites in response to long-term aerial exposure and dehydration stress when compared to hydrated control embryos at $t = 0$. The vertical dotted line separates the downregulated (left) and upregulated (right) metabolites. The color of each metabolite coordinates to the superpathway they belong to (above). A total of 18 metabolites decreased and 71 metabolites increased in both stages. Metabolites with asterisks indicate compounds that have not been confirmed based on standards, but Metabolon is confident in their identity.

Figure 4.5. Indications of altered transsulfuration activity and antioxidant utilization in DII and post-DII embryos. Box plot data illustrating metabolites contributing to methionine, cysteine, and glutathione metabolism. Data (scaled intensity) are presented as box plots with a line drawn at the median (mean indicated by a plus symbol) and the box indicating upper and lower quartiles. Bars are minimums and maximums of the distribution. Vertical dotted lines separate data from DII (blue) and post-DII embryos (orange). Data are organized by aerial exposure treatment (C, control; S, short-term; L, long-term). Asterisks represent treatments that were significantly different from the control group (Welch's two-sample t-test, $P < 0.05$). Each treatment had six biological replicates ($N = 6$) containing 25 embryos each. See Figure 4.1 for more information on sampling. Raw values for each metabolite were scaled to set the median across all samples to 1 and were normalized to DNA content. Relevant enzymes are included (grey boxes). CBS, cystathionine β -synthase; CSE, cystathionine γ -lyase; GCS, gamma-glutamylcysteine synthetase; GS, glutathione synthase; GGT, gamma-glutamyl transferase.

Figure 4.6. Changes in gamma-glutamyl amino acid metabolism in DII and post-DII embryos. Box plots illustrating metabolites involved in gamma-glutamyl amino acid metabolism that were significantly altered in at least one stage. Data (scaled intensity) are presented as box plots with a line drawn at the median (mean indicated by a plus symbol) and the box indicating upper and lower quartiles. Bars are minimums and maximums of the distribution. Vertical dotted lines separate data from DII (blue) and post-DII embryos (orange). Data are organized by aerial incubation treatment (C, control; S, short-term; L, long-term). Asterisks represent treatments that were significantly different from the control group (Welch's two-sample t-test, $P < 0.05$). Each treatment had six biological replicates ($N = 6$) containing 25 embryos each. See Figure 4.1 for more information on sampling. Raw values for each metabolite were scaled to set the median across all samples to 1 and were normalized to DNA content. Gamma-glutamylisoleucine (asterisk) had not been confirmed based on a standard, but Metabolon is confident in the identity.

Figure 4.7. Changes in creatine metabolism in DII and post-DII embryos. Box plots illustrating metabolites involved in creatine metabolism. Data (scaled intensity) are presented as box plots with a line drawn at the median (mean indicated by a plus symbol) and the box indicating upper and lower quartiles. Bars are minimums and maximums of the distribution. Vertical dotted lines separate data from DII (blue) and post-DII embryos (orange). Data are organized by aerial incubation treatment (C, control; S, short-term; L, long-term). Asterisks represent treatments that were significantly different from the control group (Welch's two-sample t-test, $P < 0.05$). Each treatment had six biological replicates ($N = 6$) containing 25 embryos each. See Figure 4.1 for more information on sampling. Raw values for each metabolite were scaled to set the median across all samples to 1 and were normalized to DNA content.

Figure 4.8. Changes in glycolysis and pentose phosphate pathway metabolism in DII and post-DII embryos. Box plots illustrating metabolites involved in glycolysis and pentose phosphate pathway (PPP) metabolism. Data (scaled intensity) are presented as box plots with a line drawn at the median (mean indicated by a plus symbol) and the box indicating upper and lower quartiles. Bars are minimums and maximums of the distribution. Vertical dotted lines separate data from DII (blue) and post-DII embryos (orange). Data are organized by aerial incubation treatment (C, control; S, short-term; L, long-term). Asterisks represent treatments that were significantly different from the control group (Welch's two-sample t-test, $P < 0.05$). Each treatment had six biological replicates ($N = 6$) containing 25 embryos each. See Figure 4.1 for more information on sampling. Raw values for each metabolite were scaled to set the median across all samples to 1 and were normalized to DNA content.

Figure 4.9. Changes in nicotinate and nicotinamide metabolism in DII and post-DII embryos. Box plots illustrating metabolites contributing to nicotinate and nicotinamide metabolism. Data (scaled intensity) are presented as box plots with a line drawn at the median (mean indicated by a plus symbol) and the box indicating upper and lower quartiles. Bars are minimums and maximums of the distribution. Vertical dotted lines separate data from DII (blue) and post-DII embryos (orange) embryos. Data are organized by aerial incubation treatment (C, control; S, short-term; L, long-term). Asterisks represent treatments that were significantly different from the control group (Welch's two-sample t-test, $P < 0.05$). Each treatment had six biological replicates ($N = 6$) containing 25 embryos each. See Figure 4.1 for more information on sampling. Raw values for each metabolite were scaled to set the median across all samples to 1 and were normalized to DNA content.

Chapter 5

GABA daba done: Summary and future directions

The ability for vertebrates to survive without oxygen for prolonged periods of time is rare and astonishing. Perhaps equally as astounding and rare is survival of aquatic vertebrates without water. Remarkably, *Austrofundulus limnaeus* experiences both of these stresses as part of their normal embryonic development. Previous work in *A. limnaeus* showed accumulation of GABA in response to anoxia. The conserved nature of GABA accumulation in all groups of anoxia-tolerant vertebrates warranted the exploration into the role of GABA in *A. limnaeus* during anoxia. Exploring GABA production and its role during anoxia promised to generate novel information regarding its role in stress tolerance of *A. limnaeus*. Although work in other anoxia-tolerant systems suggested roles for GABA during anoxia, the relationship of GABA to anoxia tolerance in *A. limnaeus* has always been speculative until this project. Perhaps the most exciting outcome of this project was being the first study to show that inhibition of GABA metabolism directly relates to survival time in anoxia. This project also revealed many aspects of survival during dehydration stress, including the first evidence of oxygen consumption rates in *A. limnaeus* embryos during aerial incubation, a portion of their lifecycle in which they undoubtedly spend substantial time. The physiological and molecular responses of *A. limnaeus* embryos to aerial incubation further expanded our knowledge of the unique biology of this system. Performing a metabolomics analysis on multiple stages of embryos in response

to dehydration stress has provided a broad assessment of the metabolites accumulated in extreme dehydration tolerance and a foundation of data for further studies.

We hypothesized that anoxia tolerance is a pre-adaptation that led to the evolution of dehydration tolerance in *A. limnaeus*, thus embryos would respond similarly at the molecular level to combat each individual stressor. There is undoubtedly overlap in the molecular responses to anoxia and dehydration stress, as demonstrated by GABA production during both stresses. However, the likelihood that GABA is serving all of the same functions during each stressor is unlikely. GABA is seen to accumulate in millimolar quantities during anoxia, a time where metabolism is severely depressed, whereas GABA likely (based on our knowledge of baseline levels and fold changes from metabolomics data) accumulates in micromolar levels during dehydration stress, a time where metabolism is high. This is not to say that micromolar concentrations are not biologically relevant, as these are the levels accumulated in other anoxia-tolerant organisms in response to anoxia, but there appears to be other major metabolic pathways at play that may further explain the extreme tolerance of embryos to dehydration stress. Although this project has elucidated various roles for GABA and uncovered other potential metabolic pathways aiding in survival during aerial incubation, there are many future studies that can be drawn from the data presented here.

GABA and lactate - multifunctional stress metabolites?

This is the first extensive study of long-term anoxia and aerobic recovery in *A. limnaeus* embryos. We investigated multiple embryological stages across development that range in their tolerance of anoxia. All developmental stages investigated responded to anoxia by accumulating significant amounts of lactate, but only those stages with greater anoxia-tolerances (DII, WS 36, and WS 40) accumulated GABA. The most anoxia-sensitive stage investigated (WS 42) failed to accumulate GABA and also failed to recover from anoxia. Our findings suggest that embryos seemingly go unharmed and without developmental defect following long-term anoxia. However, recovery from anoxia appears to be slow with lactate and GABA levels not returning to pre-anoxia levels until after at least a week of aerobic recovery. This slow reduction of both lactate and GABA may be an adaptive and protective mechanism for supporting survival of long-term anoxia.

We hypothesized that anoxia-tolerant stages would have higher basal enzymatic activity responsible for GABA production than anoxia-sensitive stages, thus being able to survive longer and produce more GABA in response to stress. However, we saw an inverse relationship between normoxic GAD activity and anoxia tolerance; embryos with the highest GAD activity (WS 42) produced negligible GABA in anoxia. Although GABA metabolism appears to be critical for long-term survival in anoxia, the innate capacity for high GAD activity does not correspond to anoxia tolerance. Through pharmacological inhibition experiments of enzymes responsible for the production (GAD) and degradation of GABA

(GABA-transaminase and succinic semialdehyde dehydrogenase), we showed for the first time that GABA metabolism affects anoxia tolerance in *A. limnaeus* embryos. Inhibition of enzymes responsible for both production and degradation of GABA led to decreased survival in the most anoxia-tolerant stage (WS 36). Interestingly, the effects of decreased GABA production on survival are not obvious until a few weeks of anoxia, suggesting a role in long-term rather than short-term survival. Gene expression patterns of an abundance of GABA and lactate transporters in *A. limnaeus* embryos support the capability for intraembryonic or intercellular shuttling of lactate and GABA. Together, our results support a role for GABA as an intermediate product and not a metabolic end product. We proposed multiple roles for GABA during anoxia and aerobic recovery in *A. limnaeus* embryos: serving as a neurotransmitter, an energy source, and an antioxidant. Recent advancements in non-metabolic functions of GABA and lactate have shown they both can act as inhibitors of histone deacetylase (HDAC), and that lactate can serve to increase transcription through lactylation of histones (Xing et al., 2019; Zhang et al., 2019). GABA and lactate both gradually decrease during aerobic recovery, which could be a coordinated effort to help return gene expression patterns back to normal.

Although GABA and lactate may not be accumulating at levels seen in anoxia, they could be serving several important and similar functions during dehydration. Even at the micromolar range, GABA and lactate can function as important neuroprotective agents. The accumulation of other neuroprotective compounds during dehydration further enforces the need for *A. limnaeus*

embryos to protect themselves from neural injury. As previously mentioned, both of these metabolites can function as epigenetic modifiers. Epigenetic changes through methylation and histone modifications can help cells control expression of genes by precisely turning them on or off, which could help explain the abundant changes in metabolite profiles we see in response to dehydration stress. Embryos also accumulate 2-hydroxyglutarate, a known epigenetic modifier, which further suggests a role for metabolite-driven control of gene expression in survival. Deducing the specific functions of GABA and lactate are playing in response to dehydration requires further studies, but it is clear there are several promising functions which could aid in their survival.

Terrestrial eggs in an aquatic vertebrate?

Despite knowledge of the unprecedented ability of annual killifish embryos to survive long periods of aerial exposure, this is the first time that dehydration tolerance has been measured under controlled conditions across development. We show the remarkable ability for DII embryos to survive over a year and a half without water, exhibiting a lethal time to 50% mortality (LT_{50}) of 325 d. Despite having similar anoxia tolerance to DII embryos, WS 36 embryos *only* survive a few months without water, exhibiting an LT_{50} of 84 d. Even the most sensitive stages to dehydration stress (WS 40 and WS 42) were able to survive 100 d without water, exhibiting an LT_{50} of 28 and 29 d, respectively. Perhaps most remarkable is the ability for embryos of this aquatic vertebrate to survive in terrestrial environments for as long as that of amniotic terrestrial eggs. In

addition, we measured oxygen consumption rates of embryos incubated in aqueous and aerial conditions. Embryos in DII respond to dehydration stress by increasing rates of oxygen consumption. On the other hand, post-diapause II embryos either exhibit the same or reduced rates of oxygen consumption when compared to aqueous embryos. Aerial incubation coupled with hypoxia causes some embryos to arrest development. The stage-specific response of annual killifish embryos to dehydration stress are consistent with an intrinsic bet-hedging strategy in embryos that would increase developmental variation in a potentially adaptive manner. The extreme tolerance of DII embryos to dehydration stress observed suggest it is likely the stage responsible for survival of *A. limnaeus* populations during the unpredictable dry seasons.

A major focus of this thesis was to gain insight into the mechanisms facilitating survival during aerial exposure by examining the metabolite profiles of dormant and actively developing embryos. There is strong evidence for differences in metabolic profiles in the study based on developmental stage and length of aerial exposure. Actively developing embryos exhibit more robust change, however, dormant embryos respond in an active manner by significantly altering their metabolic profile. We identify a number of metabolites that accumulate in both embryos that may be playing an important role in survival, including the identification of known antioxidants and neuroprotectants. In addition, we identify unique metabolites not yet discussed in the dehydration literature, such as the non-proteinogenic amino acid lanthionine and oncometabolite 2-hydroxyglutarate. Despite high oxygen availability, embryos

accumulate the anaerobic glycolytic end-product lactate and neurotransmitter GABA. These metabolites may be acting as extracellular signaling molecules through the activation of G-protein-coupled receptors (GPCRs) (Husted et al., 2017). Together, this paper offers an overview of the metabolic changes occurring that may support embryonic survival during dehydrating conditions, which can be functionally tested using genetic and pharmacological approaches.

Embryos of *A. limnaeus* likely spend a majority of their life span aerially incubated and thus must deal with waste products in a unique way. During aerial incubation, embryos are unable to shuttle waste to the outside environment or even into the perivitelline space, as all fluid is void. Instead, embryos most likely rely on mechanisms very similar to those of amniotic eggs, which is to transport waste away from the developing embryo into extraembryonic compartments, such as the yolk sac or the extraembryonic fluid. In essence, the yolk sac and extraembryonic fluid serve analogous roles with the allantois and amniotic fluid of amniotes. The extraordinary ability for embryos to survive terrestrial conditions paired with potential waste management mechanisms analogous to amniotic eggs suggest *A. limnaeus* may represent an intermediate in the evolution of amniotes. This is further bolstered by *A. limnaeus* embryos accumulating many compounds that are usually found in the amniotic and allantoic fluid in terrestrial species. Further studies into the actions of these compartments during aerial incubation might lead to interesting discoveries on the waste regulation and evolution of this complex system.

Future research directions

One of the most interesting results from this thesis was the observation of decreased survival of embryos in anoxia when production *and degradation* of GABA was inhibited. This observation was shown in the anoxia-tolerant and metabolically active WS 36 embryos. It would be interesting to see if similar results are observed in dormant DII embryos that exhibit a similar anoxia tolerance. We showed that GABA appears to be aiding in long-term survival, so it would be interesting to see if inhibition of GABA production or degradation would reduce the already short anoxia tolerance of WS 40 embryos ($LT_{50} = 7$ d). Supplementation of GABA into the embryo medium to potentially extend the tolerance of anoxia-sensitive embryos may seem like a logical next step. However, the enveloping cell layer of *A. limnaeus* embryos appears to be nearly impermeable to amino acids even when using a vehicular aid such as DMSO. Thus, it is unlikely that GABA would permeate in sufficient amounts. A possible next step is to test this hypothesis using embryo-derived anoxia-tolerant cell lines. There are currently two cell lines of neural origin derived from embryos of *A. limnaeus* (PSU-AL-WS40NE and WS36-2) that can survive for more than 49 days without oxygen. A cell model would allow us to easily treat cells with GABA to determine if GABA supplementation extends anoxia tolerance. This would be especially interesting in these cell lines as they have not been seen to accumulate GABA despite surviving for so long in anoxia. Further, we can compare the effects of GABA on anoxia-sensitive cell lines derived from mouse, monkey, or human to see if we can extend their tolerances.

A difficulty with measuring metabolites in whole embryos is accounting for the many compartments in which these metabolites can exist in (e.g., perivitelline fluid, embryonic tissue, yolk sac, and the extraembryonic cavity bounded by the enveloping cell layer). We suggest the possibility that GABA and lactate are being shuttled from the embryonic tissue to the yolk sac, thus localization of these metabolites is an important next step. Further studies into GABA and lactate presence in yolk and cellular fractions of embryos will help elucidate the role the yolk may be playing as a buffering or storage system. We observed many gene transcripts associated with GABA and lactate transporters, as well as GABA receptors, though we do not know their function or localization during anoxia or normal development. Immunohistochemistry would allow for localization of these proteins as well as localization of GABA and lactate themselves, which would further illuminate our understanding of anoxia survival. We also suggested the slow decrease in GABA and lactate during aerobic recovery from anoxia may be mechanisms for mitigating reactive oxygen species (ROS) production, thus investigating ROS levels in embryos as it relates to GABA and lactate levels during aerobic recovery will also be an important next step.

The work in this thesis revealed differences in the rate of post-DII development in embryos during dehydration exposure. When oxygen was limited, but still above the measured P_{crit} of *A. limnaeus* embryos, aerial incubation led to developmental arrest in a proportion of embryos. However, post-DII embryos developing with ample oxygen (in large desiccators) develop

synchronously. Beyond the evidence in this dissertation, little is known about development during aerial exposure and what controls whether an embryo will arrest development. Anecdotal evidence shows embryos that break DII while on filter pads, though uncommon, develop drastically slower than embryos that are already post-DII when placed on filter pads. Although it appears as though embryos develop unharmed during aerial incubation, further research needs to be done to examine embryos and their morphology as they develop in aerial conditions.

During this dissertation work, embryos were never rehydrated following long-term dehydration, thus we do not know how embryos will react following reintroduction of water. From a developmental and ecological perspective, it would be interesting to see if reintroduction of water to DII embryos causes them to break DII and continue developing. This would mimic what we assume they experience in the wild and thus a normal part of their life cycle. As we know there are a lot of dramatic changes in cell physiology occurring during transition from aqueous to aerial conditions, there undoubtedly are changes occurring when going from a dehydrated state back to hydrated. Going forward, it could be of interest to examine how the metabolome of dehydrated embryos responds upon rehydration. Now that we have a foundation of metabolites to compare to, we can perform targeted assays to measure the abundance of certain metabolites during aerial incubation and rehydration that we believe may be playing key roles in survival.

Continued research on non-model organisms holds promise for better understanding how organisms have adapted to overcome physiological challenges imposed by environmental stress. Since many of the mechanisms utilized by *A. limnaeus* embryos in response to anoxia and dehydration stress are conserved across taxa, it is likely that these same mechanisms can be exploited in sensitive models, such as humans, which could lead to promising medical applications.

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Appendix: Supplemental File Information

File	Name	File size	File type	Software
Supplemental Table S4.1	All metabolites identified in embryos of <i>Austrofundulus limnaeus</i> exposed to dehydration stress due to aerial incubation.	344 KB	xlsx	Microsoft Excel