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GABA metabolism is crucial for long-term survival of anoxia in annual killifish embryos

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Summary Statement

Anoxic annual killifish embryos produce millimolar quantities of GABA. Interruptions in GABA metabolism decrease anoxia tolerance. We propose that GABA supports anoxia tolerance as a neurotransmitter and an antioxidant.

Abstract

In most vertebrates, a lack of oxygen quickly leads to irreparable damages to vital organs, such as the brain and heart. However, there are some vertebrates that have evolved mechanisms to survive periods of no oxygen (anoxia). The annual killifish (*Austrofundulus limnaeus*) survives in ephemeral ponds in the coastal deserts of Venezuela and their embryos have the remarkable ability to tolerate anoxia for months. When exposed to anoxia, embryos of *A. limnaeus* respond by producing significant amounts of γ-aminobutyric acid (GABA). This study aims to understand the role of GABA in supporting the metabolic response to anoxia. To explore this, we investigated four developmentally distinct stages of *A. limnaeus* embryos that vary in their anoxia tolerance. We measured GABA and lactate concentrations across development in response to anoxia and aerobic recovery. We then inhibited enzymes responsible for the production and degradation of GABA and observed GABA and lactate concentrations, as well as embryo mortality. Here, we show for the first time that GABA metabolism affects anoxia tolerance in *A. limnaeus* embryos. Inhibition of enzymes responsible for GABA production (glutamate decarboxylase) and degradation (GABA-transaminase and succinic acid semialdehyde dehydrogenase) led to decreased mortality, supporting a role for GABA as an intermediate product and not a metabolic end product. We propose multiple roles for GABA during anoxia and aerobic recovery in *A. limnaeus* embryos, serving as a neurotransmitter, an energy source, and an antioxidant.

Abbreviations used:

DAO = diamine oxidase

DEAB = N,N-diethylaminobenzaldehyde

DII = diapause II

DMSO = dimethyl sulfoxide

dpd = days post-diapause II

dpf = days post fertilization

EKP = ethyl ketopentenoate

FPKM = fragments per kilobase per million mapped reads

GABA = γ-aminobutyric acid

GABA-T = GABA transaminase

GAD = glutamate decarboxylase

GAT = GABA transporter

LDH = lactate dehydrogenase

LT_{50} = lethal time to 50% mortality

MCT = monocarboxylate transporter

ROS = reactive oxygen species

SSADH = succinic acid semialdehyde dehydrogenase

SSAL = succinic semialdehyde

WS = Wourms' stage

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, 1972a; Wourms, 1972b). 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 (Boutilier, 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 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} $MgCl_2$, 0.8 mmol l^{-1} $CaCl_2$, 0.14 mmol l^{-1} KCl, 1.3 mmol l^{-1} $MgSO_4$) (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., 2010; Romney et al., 2018). Embryos enter DII at around 24 dpf at 25ºC.

Developmental staging and sampling of embryos

Diapause II was broken by subjecting embryos to a temperature of 30ºC 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, 1972a; Wourms, 1972b). 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 −80ºC 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 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% CO_2, 5% H_2, and 90% N_2 with temperature held constant at 25ºC 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 N_2 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 3A). 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 µmol l^{-1} EKP; 1 mmol l^{-1} Vigabatrin; 167 µmol 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.

Metabolite extraction

Metabolites were extracted from whole embryos following previously detailed protocols (Podrabsky and Hand, 1999) and briefly described here. Samples were removed from −80ºC 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 ***g*** for 15 min at 4ºC 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 ***g*** for 30 min at 4ºC. Supernatants were then transferred to new tubes and frozen at −80ºC 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 µl) 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 µl 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 ± 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 ± 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 −80ºC 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 µmol 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 4ºC. Supernatants containing GAD were removed and frozen at −80ºC 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 −80ºC 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 4ºC 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 25ºC 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 −80ºC 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 25ºC 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 20ºC 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 25ºC 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). LT_{50} 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 regression 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 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 1B; Table 1). Initial rates were higher than final rates for each stage of embryo (Table 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 1B; 23.6 ± 1.09 mmol kg^{-1} embryo; mean ± 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 1). We observed a significant correlation between initial rates of lactate accumulation and LT_{50} in anoxia (Fig 2A). The relationship between initial rates of lactate accumulation and LT_{50} in anoxia reported here on DII and post-DII embryos is similar to those from previous experiments on pre-DII and DII embryos (Fig 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 42; 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 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 1B; Table 1). Regression analysis of data for WS 42 embryos confirmed a slope that was not statistically different from zero (Table 1). Initial rates of GABA accumulation were higher than final rates in DII and WS 36 embryos (Table 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 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 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 µmol l^{-1}), GABA-T inhibitor vigabatrin, and SSADH inhibitor DEAB survived, developed normally, and had developmental rates that were indistinguishable from those of control embryos (data not shown). In contrast, embryos exposed to 800 µmol l^{-1} EKP showed mortality following 5 d of exposure (Fig S1). However, it should be noted that embryos continue developing in normoxia, which is not the case during exposure to anoxia. Additionally, embryos survived in anoxia at WS 36 significantly longer than developing embryos exposed to 800 µmol l^{-1} EKP in normoxia (Fig 4B,C; Fig S1). Exposure to 800 µmol l^{-1} EKP starting at WS 40 led to mortality after 2 d (Fig S1). 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 3B,C). GABA levels were significantly lower after 14 d in 400 µmol l^{-1} treated embryos when compared to controls, while levels were significantly lower after only 7 d in 800 µmol l^{-1} treatments (Fig 3B,C; two-way ANOVA with Tukey's HSD, $P < 0.05$). Embryos exposed to 400 µmol l^{-1} EKP produced GABA at a rate 42% slower than control embryos; whereas, embryos exposed to 800 µmol 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 3D,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 4A,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 ± SEM, $N = 3$) in anoxia for 400 and 800 µmol l^{-1} treatments, respectively (Fig 4C). Despite having similar mean LT_{50} values (one-way ANOVA, $P = 0.78$), 800 µmol l^{-1} treated embryos did not survive as long as 400 µmol l^{-1} treated embryos (Fig 4B,C). Embryos treated with 400 µmol l^{-1} reached 100% mortality after 62 d, while it only took 51 d for 800 µmol 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 3C; 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 3E; 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 ± 0.5 d; mean ± SEM, $N = 3$; Fig 4B,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 3B, 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 3D; two-way ANOVA with Tukey's HSD, $P > 0.05$; ANCOVA, $P > 0.05$). DEAB decreased survival of anoxic embryos (Fig 4B), leading to a 44% reduction in LT_{50} (39 ± 4 d; mean ± SEM, $N = 3$; Fig 4C).

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 S2; regression analysis, $P < 0.005$). We observed low, but significantly greater than zero, rates of GAD activity in post-DII embryo homogenates (Fig 5A). No significant GAD activity was detected in DII embryos after 24 h of incubation (Fig 5A). 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 5B; 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 1B). There is a significant negative relationship between LT_{50} and GAD activity (Fig 5C).

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 6A). 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 5C) 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 6B). 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 6C). 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 6C; *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 6D). 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 6D). 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 7). 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 $GABA_A$R 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 $GABA_A$R 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 7). 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 7). 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 S3A; $R^2 = 0.07$, $P = 0.67$). Interestingly, embryos from younger females produced lactate during anoxia at a faster rate than embryos produced by older females (Fig S3B; $R^2 = 0.97$, $P < 0.01$).

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 LT_{50}s) 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 LT_{50}, 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 transporter transcripts expressed in *A. limnaeus* embryos suggest GABA may be shuttled within the embryo or within cells after production. It is important to note that this would only be the case if the proteome matches the transcript data. The expression of high levels of GABA transporter transcripts in DII embryos and anoxia-induced elevation in transcripts of GABA transporters in WS 36 embryos suggests 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). If the transcript data reflect the proteome, then these 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. 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.

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Competing interests

The authors declare no competing interests.

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Table 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[a] (mmol kg^{-1} $embryo^{-1}$ d^{-1})	0.126	0.281	0.561	[b]
	X_0 (d)	16	18	2	[b]
	Final[a] (mmol kg^{-1} $embryo^{-1}$ d^{-1})	0.035	0.156	0.733	[b]
	Degrees of Freedom	16	16	13	[b]
	R^2	0.97	0.97	0.96	[b]
Lactate	Initial[a] (mmol kg^{-1} $embryo^{-1}$ d^{-1})	0.152	0.497	3.679	26.83
	X_0 (d)	16	18	2	0.17
	Final[a] (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] Initial and final rates of accumulation were calculated from data presented in Figure 1B.
[b] 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).

Figures

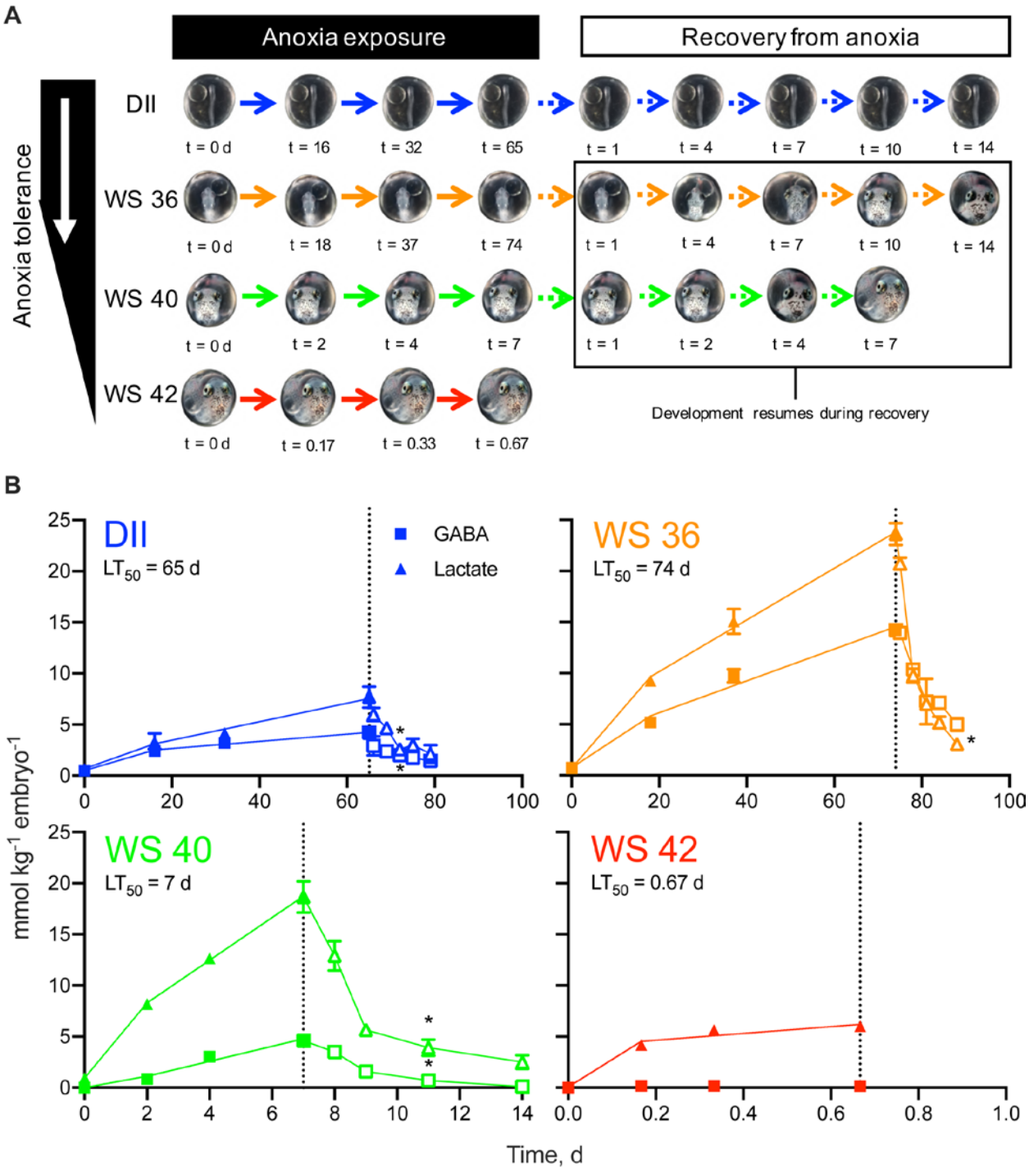


Figure 1. GABA and lactate accumulation in embryos of *Austrofundulus limnaeus* during exposure to anoxia and aerobic recovery from anoxia at 25°C. (**A**) A schematic showing the experimental design and sampling regimen used. Embryos from 4 developmental stages were

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 recovery, 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 ± 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 1 for results of regression analyses.

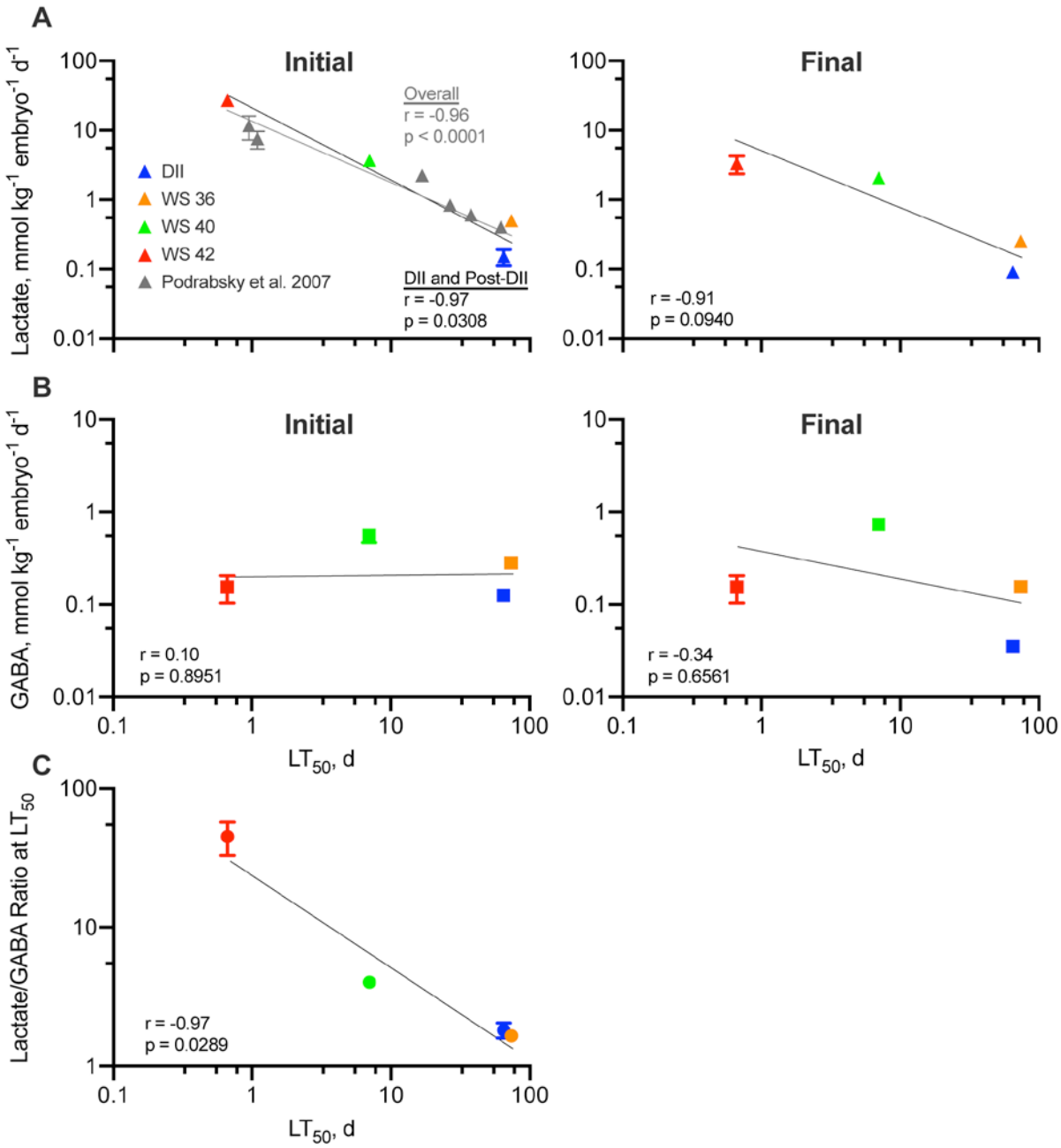


Figure 2. The relationship between lethal time to 50% mortality (LT_{50}) and anoxic GABA and lactate accumulation in embryos of *A. limnaeus*. (**A**) LT_{50} was highly correlated with initial rates of lactate accumulation in anoxia in embryos of *A. limnaeus* (Correlation analysis, $P < 0.05$). When 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 ± 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.

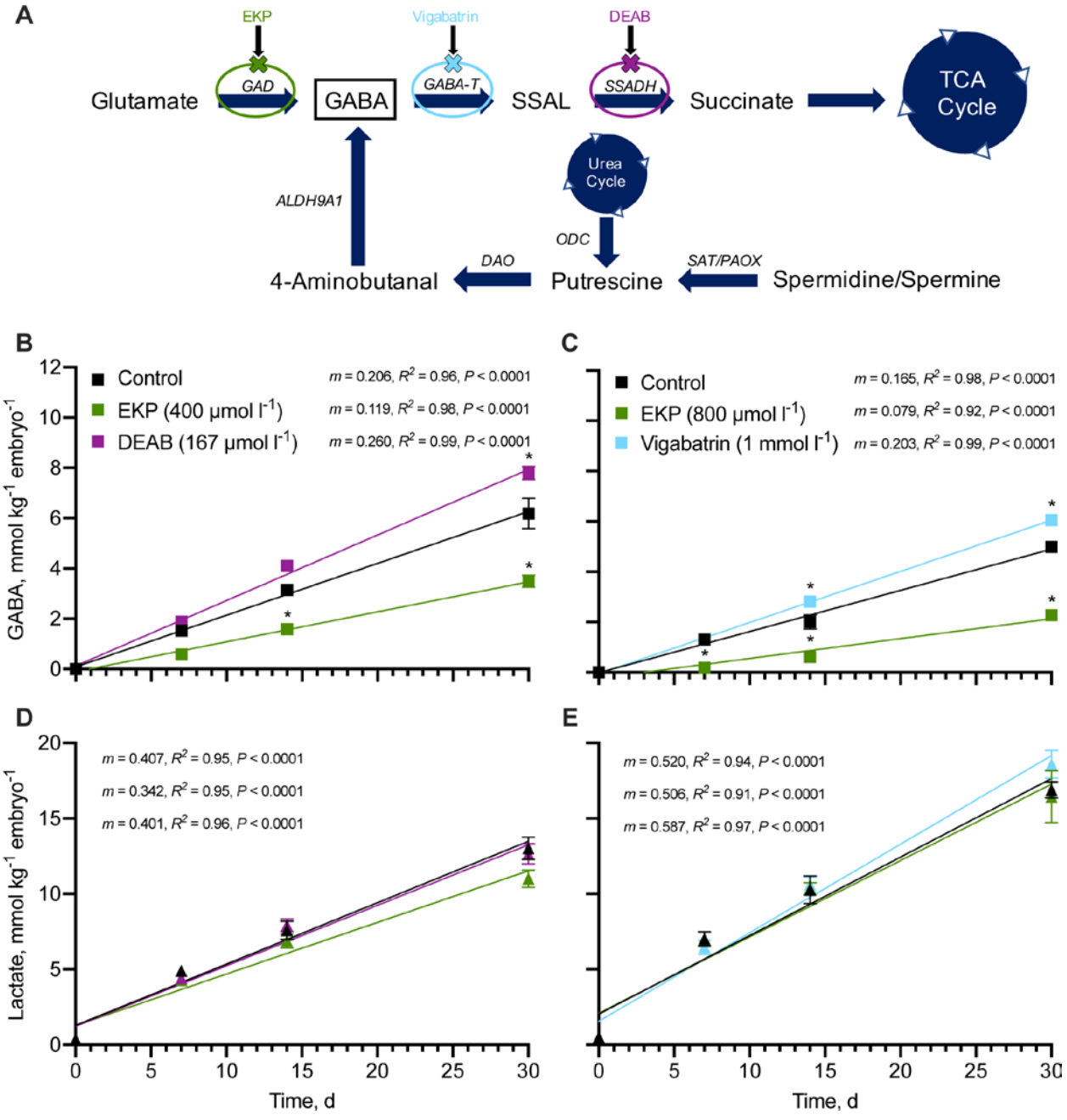


Figure 3. GABA and lactate accumulation during inhibition of the GABA synthesis and degradation pathway in Wourms' stage 36 embryos exposed to anoxia. (**A**) A schematic depicting canonical and non-canonical synthesis and metabolic degradation of GABA and the key enzymes pharmacologically inhibited 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 ± 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, $P > 0.05$).

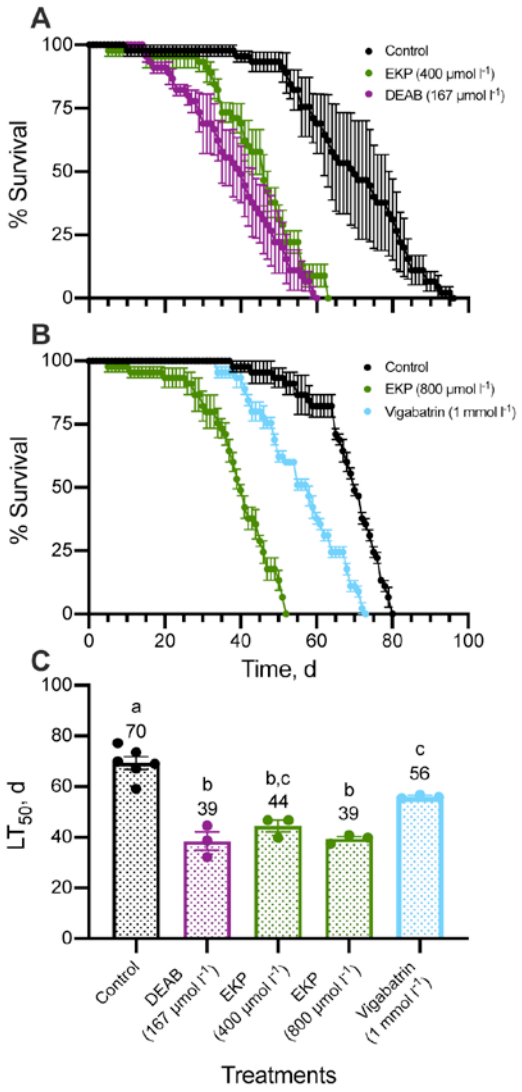
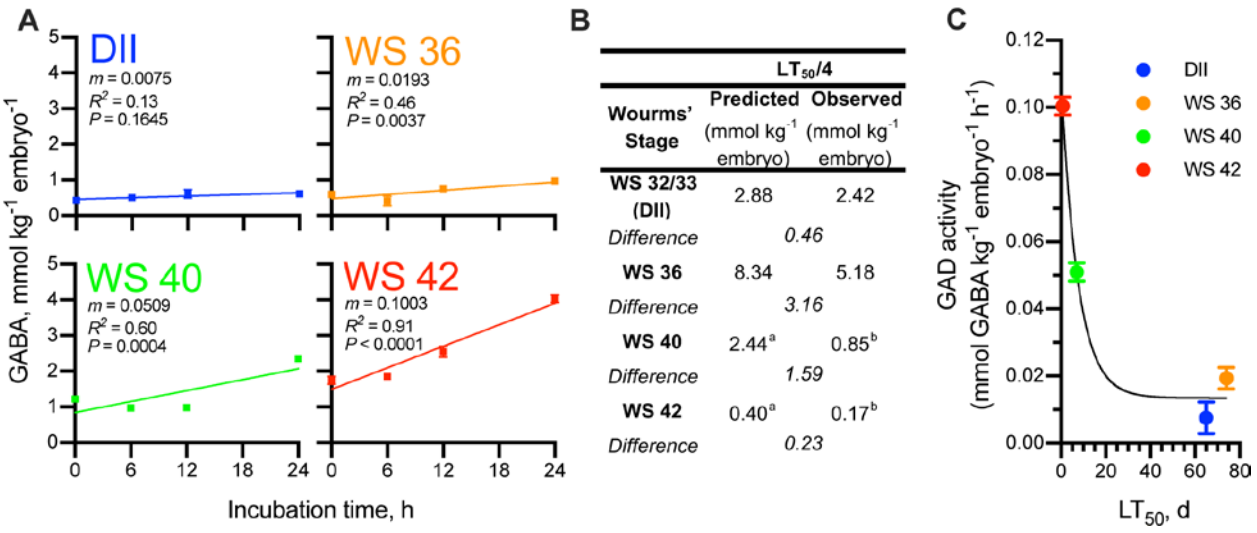


Figure 4. Survival of Wourms' stage 36 embryos continuously exposed to GAD inhibitor EKP, SSADH inhibitor DEAB, and GABA-T inhibitor vigabatrin in anoxia. Groups of 15 embryos were exposed to embryo medium containing (**A**) 400 µmol l^{-1} EKP or 167 µmol l^{-1} DEAB, (**B**) 800 µmol l^{-1} EKP, or 1 mmol l^{-1} vigabatrin in anoxia at 25°C. Symbols are means ± SEM (*N* = 3). (**C**) Lethal time to 50% mortality (LT_{50}) for embryos exposed to anoxia. Different letters indicate significant differences in LT_{50} between treatments (one-way ANOVA with Tukey's HSD test, $P < 0.05$). Bars are means ± 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.



Wourms' Stage	$LT_{50}/4$ Predicted (mmol kg^{-1} embryo)	$LT_{50}/4$ Observed (mmol kg^{-1} embryo)
WS 32/33 (DII)	2.88	2.42
Difference	*0.46*	
WS 36	8.34	5.18
Difference	*3.16*	
WS 40	2.44[a]	0.85[b]
Difference	*1.59*	
WS 42	0.40[a]	0.17[b]
Difference	*0.23*	

Figure 5. 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 ± 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 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 LT_{50} 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 ± SEM ($N = 4$).

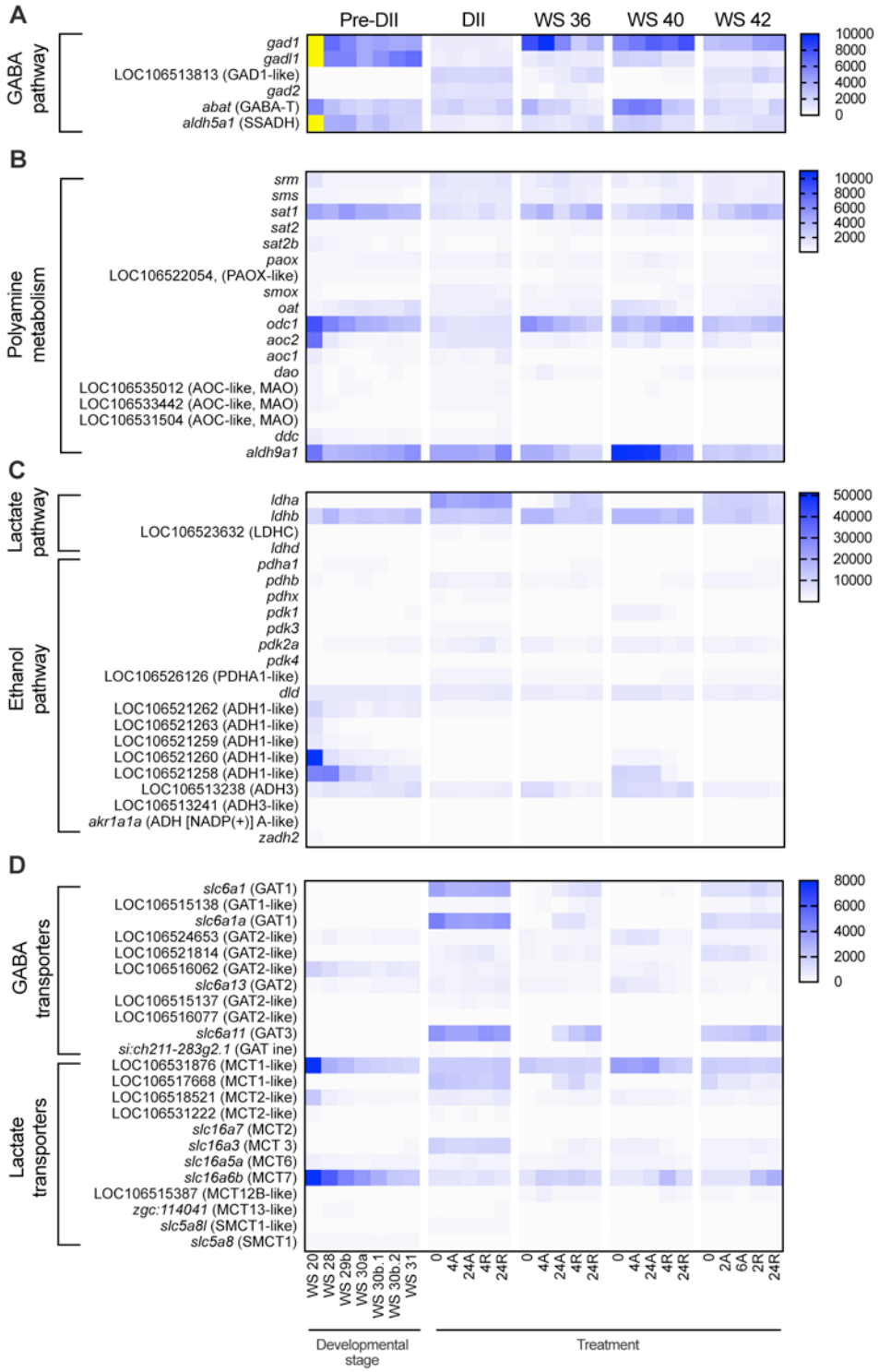


Figure 6. Transcript levels for GABA, polyamine, lactate, and ethanol pathways in *A. limnaeus* as determined by RNA sequencing. Enzyme systems are organized into groups: (**A**) GABA synthesis and degradation, (**B**) polyamine metabolism, (**C**) lactate and ethanol synthesis, and (**D**) GABA and lactate transportation. 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. Cells in yellow indicate transcript levels that were overexpressed (FPKM = 11,000−30,000).

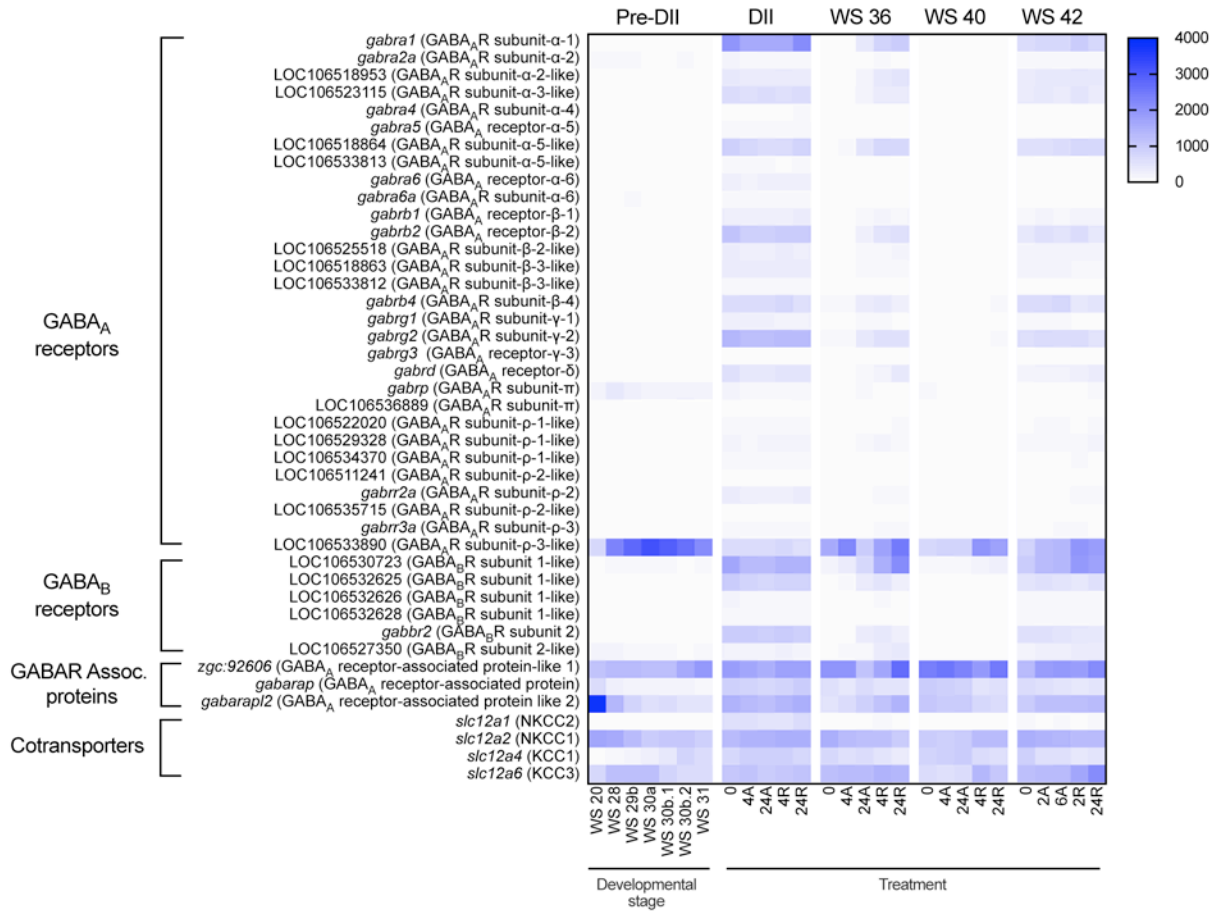


Figure 7. Transcript levels for GABA signaling and cotransporters in *A. limnaeus* as determined by RNA sequencing. Enzyme systems are organized into groups: $GABA_A$ receptors and subunits, $GABA_B$ receptors and subunits, GABA receptor associated proteins, and Na^+-K^+-Cl^- (NKCC) and K^+-Cl^--cotransporters (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.

Supplemental Figures

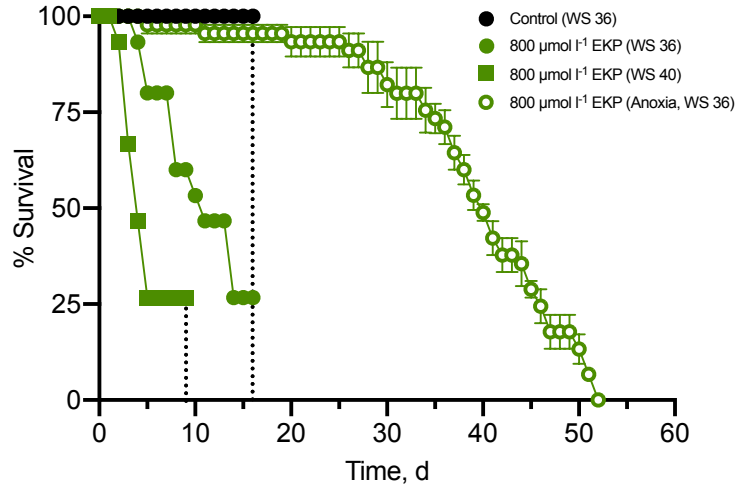


Figure S1. **Survival of embryos to 800 µmol l^{-1} ethyl ketopentenoate (EKP) in normoxia and anoxia.** Wourms' stage (WS) 36 embryos exposed to EKP in anoxia survived longer than developing embryos (WS 36 and WS 40) exposed to EKP in normoxia. WS 40 embryos exhibited mortality after 2 d of exposure, 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 ± SEM of groups of 15 embryos (*N* = 3) and are from Figure 4 of the main manuscript and are provided here for ease of comparison.

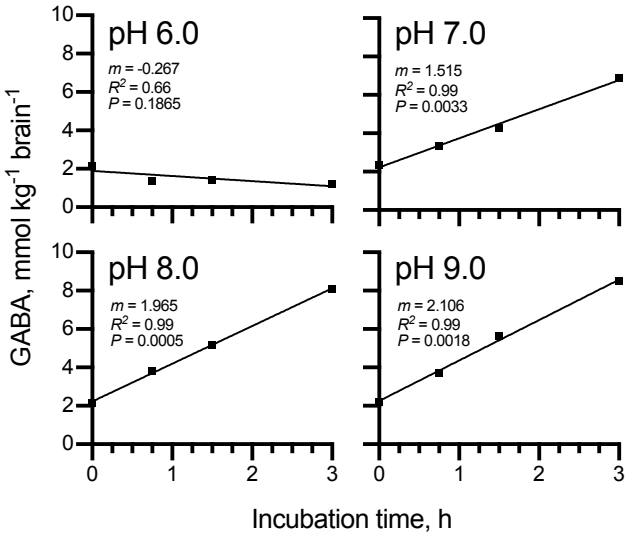


Figure S2. Glutamate decarboxylase (GAD) activity in adult male *A. limnaeus* brain homogenates in normoxia at 25°C. Adult brain homogenates generate GABA that are significantly greater than 0 at pH 7.0, 8.0, and 9.0 (Regression analysis, $P < 0.005$), while pH 6.0 homogenates did not show a significant 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.

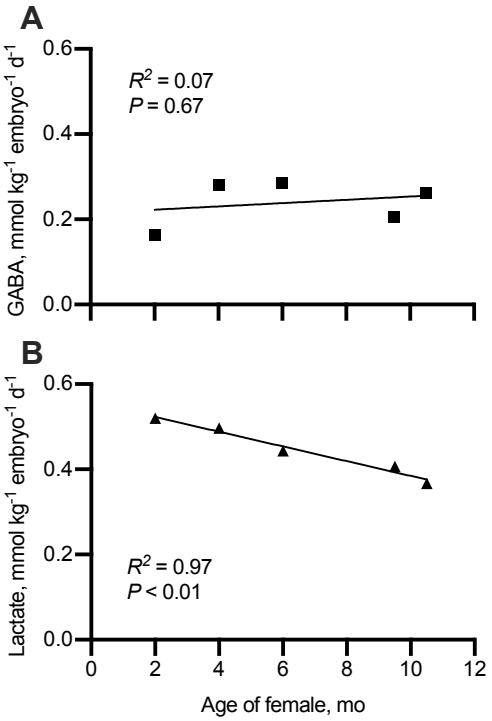


Figure S3. The relationship between female age and rates of GABA and lactate accumulation in anoxic Wourms' stage 36 embryos. (**A**) The rate of GABA accumulation in embryos did not correlate with female age; however (**B**) there was a significant negative linear relationship in the rate of lactate 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.