The Long-Term Effects of Developmental Hypoxia on Cardiac Mitochondrial Function in Snapping Turtles

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It is well established that adult vertebrates acclimatizing to hypoxic environments undergo mitochondrial remodeling to enhance oxygen delivery, maintain ATP, and limit oxidative stress. However, many vertebrates also encounter oxygen deprivation during embryonic development. The effects of developmental hypoxia on mitochondrial function are likely to be more profound, because environmental stress during early life can permanently alter cellular physiology and morphology. To this end, we investigated the long-term effects of developmental hypoxia on mitochondrial function in a species that regularly encounters hypoxia during development—the common snapping turtle (Chelydra serpentina). Turtle eggs were incubated in 21% or 10% oxygen from 20% of embryonic development until hatching, and both cohorts were subsequently reared in 21% oxygen for 8 months. Ventricular mitochondria were isolated, and mitochondrial respiration and reactive oxygen species (ROS) production were measured with a microrespirometer. Compared to normoxic controls, juvenile turtles from hypoxic incubations had lower Leak respiration, higher P:O ratios, and reduced rates of ROS production. Interestingly, these same attributes occur in adult vertebrates that acclimatize to hypoxia. We speculate that these adjustments might improve mitochondrial hypoxia tolerance, which would be beneficial for turtles during breath-hold diving and overwintering in anoxic environments.

Keywords: heart, metabolism, reptile, developmental programming, reactive oxygen species, electron transport chain, oxygen affinity, developmental plasticity

INTRODUCTION

Low O₂ availability (hypoxia) is a common environmental stressor in aquatic, terrestrial, and subterranean environments (Bickler and Buck, 2007). Hypoxia can have severe metabolic consequences for animals, because O₂ plays a pivotal role in the production of ATP and reactive oxygen species (ROS) during oxidative phosphorylation (Mitchell, 2011). Briefly, electrons derived from carbohydrates, fats, and proteins are transferred to the mitochondrial electron transport chain, where they move through a series of complexes and finally bind to O₂ at complex IV. The energy released from the transfer of electrons is utilized by complexes I, III, and IV to pump protons against
their electrochemical gradient and establish a proton-motive force that drives ATP production through complex V (the F}_{1}F_{0} ATP-synthase) (Papa et al., 2012). Although most electrons complete this journey, a small proportion slip from the chain and bind directly to molecular O_{2} to produce superoxide (Brand, 2016). When O_{2} becomes limiting, the electron transport chain is inhibited and electron slip becomes more common, leading to reduced ATP production and the overproduction of ROS (Solaini et al., 2010). Consequently, prolonged periods of hypoxia are associated with energy depletion and oxidative stress, which can ultimately lead to cell death.

Despite the profound metabolic consequences of hypoxia, many animals successfully exploit hypoxic environments (Bickler and Buck, 2007). Ectothermic vertebrates are particularly hypoxia-tolerant, with many species inhabiting aquatic and subterranean environments, where O_{2} can become severely limited or even completely absent (anoxia) (Bickler and Buck, 2007; Richards, 2009). These organisms have evolved a suite of metabolic adaptations that compensate for O_{2} deprivation (Richards, 2009). For example, acclimatization to hypoxia is commonly associated with metabolic rate suppression, a downregulation of oxidative phosphorylation, and the activation of anaerobic pathways (Richards, 2009). Central to this strategy is a structural and functional remodeling of the mitochondria (Fuhrmann and Brune, 2017; Sokolova, 2018) that usually involves a reduction in mitochondrial content and a downregulation of enzymes involved in oxidative phosphorylation (Johnston and Bernard, 1982; Donohoe and Boutilier, 1998; St-Pierre et al., 2000a; Heather et al., 2012; Hickey et al., 2012; Murray and Horscroft, 2016). Although these modifications reduce ATP production, the strategy serves to limit ROS production and oxidative stress, which can have disastrous consequences for cellular survival (Brand, 2016). The adaptive nature of this response is also supported by studies that show mitochondrial content and aerobic capacity are reduced in species that are genetically adapted to hypoxia, such as human populations at high altitude (Horscroft et al., 2017) and hypoxia-adapted fly strains (Ali et al., 2012). To overcome the decline in oxidative capacity, some vertebrates improve the efficiency of oxidative phosphorylation by modifying aspects of the electron transport chain. For example, humans acclimatized to high altitude have lower levels of uncoupling proteins and proton leak, leading to an improvement in mitochondrial coupling efficiency (the amount of ADP phosphorylated per oxygen consumed) (Jacobs et al., 2012; Levet et al., 2012). Similarly, different isoforms of complex-IV subunits can be preferentially expressed under hypoxic conditions, resulting in a higher complex-IV turnover rate and reduced oxygen affinity (Sinkler et al., 2017; Pajuelo Reguera et al., 2020). In aggregate, these studies demonstrate that mitochondria are capable of remarkable phenotypic plasticity and play an essential role in hypoxic survival.

Although the effects of hypoxia on mitochondrial function in vertebrates are well studied in adulthood, far less is known about embryonic life stages. Among ectotherms, oviparous species are particularly prone to hypoxic episodes during embryonic development (Lutz and Dunbar-Cooper, 1984; Booth, 1998; Wu, 2009; Podrabsky and Wilson, 2016). Several studies have shown that chronic hypoxia leads to an active suppression of embryonic oxygen consumption in a range of ectotherms, including fish (Gnaiger et al., 1987; Miller et al., 2008; Lindberg and Di Giulio, 2019; Wood et al., 2019), frogs (Bradford and Seymour, 1988), turtles (Kam, 1993), and crocodiles (Booth, 2000). Furthermore, exposure to hypoxia increases embryonic hypoxia tolerance by lowering critical oxygen tensions (P_{CrH} in snapping turtles, zebrafish, and salmon (Kam, 1993; Barrionuevo et al., 2010; Del Rio et al., 2019; Wood et al., 2019). The reduction in embryonic metabolism and improvement in P_{CrH} could be driven, at least in part, by mitochondrial remodeling. Indeed, similar to adult vertebrates, chronic hypoxia leads to a reduction in embryonic mitochondrial content and aerobic capacity in zebrafish (Lindberg and Di Giulio, 2019; Laquatra et al., 2021), which might contribute to metabolic suppression. Importantly, mitochondrial remodeling during embryonic development could have profound consequences for ectothermic vertebrates, because adaptive responses during early life can often be permanent, leading to life-long changes in morphology, physiology, and behavior (West-Eberhard, 2003). Nevertheless, to our knowledge, only one study has investigated the long-term impact of developmental hypoxia on mitochondrial function in an ectothermic vertebrate. In that study from our laboratory, cardiac mitochondria from juvenile alligators that were previously exposed to developmental hypoxia had lower levels of proton leak and higher respiratory-control ratios, which indicate enhanced mitochondrial efficiency (Galli et al., 2016). This is an interesting finding, because it suggests that embryonic exposure to hypoxia can program mitochondrial traits in ectothermic vertebrates that might be beneficial for hypoxic survival in adulthood. To explore this hypothesis further, the present study investigated the long-term impact of developmental hypoxia on mitochondrial function in the common snapping turtle, Chelydra serpentina.

Snapping turtles bury their eggs in subterranean nests that regularly become hypoxic, due to the combined changes in environmental gas conductance, rising egg-mass metabolism, and the metabolic activity of microorganisms (Papa et al., 2012; Brand, 2016). The extent of hypoxia in turtle nests is variable and depends on nest shape and egg location, but some species can be subjected to levels of O_{2} as low as 11% of air saturation (Papa et al., 2012). Programming of hypoxia-tolerant traits would be particularly beneficial for snapping turtles, because this species regularly encounters hypoxic environments during adulthood, and they overwinter in anoxia for up to 5 months in ice-covered lakes (Uitlsh, 2006). Importantly, laboratory studies have shown that juvenile and adult snapping turtles from hypoxic incubations have altered cardiac structure and function (Eme et al., 2013, 2014; Tate et al., 2015; Wearing et al., 2016, 2017), lower levels of basal ROS production, and improvements in cardiomyocyte anoxia tolerance (Ruhr et al., 2019). These changes are likely to involve adjustments in mitochondrial function. Therefore, we hypothesized that developmental hypoxia programs snapping turtle mitochondrial aerobic capacity and ROS production in a manner that protects the heart from hypoxic or anoxic injury. In support of our
hypothesis, we found that developmental hypoxia improved the efficiency of ATP production, as well as reducing basal levels of ROS production.

MATERIALS AND METHODS

Turtle Collection, Incubation, and Husbandry

Snapping turtle (C. serpentina) eggs were collected from the wild in Minnesota, United States, and transported to the University of North Texas for incubation (altitude elevation = 70 m and barometric pressure 759–760 mmHg). Permission to collect the eggs was granted to DA Crossley by the Minnesota Department of Natural Resources (permit no. 21232). Two eggs from individual clutches were staged to determine age. Incubations lasted no more than 55 days and all eggs were maintained at the sex-determining temperature of 30°C, to ensure all embryos developed as females (Alvine et al., 2013). Eggs were embedded to their midpoint in vermiculite, inside plastic incubators (2.5-l Ziploc® Container, SC Johnson, Racine, WI, United States) that were stored in a walk-in Percival Environmental Control Room (model IR-912L5; Percival Scientific, Perry, IA, United States). The vermiculite was mixed in a 1:1 ratio with water, as previously described (Crossley and Altimiras, 2005).

At approximately 20% development (9–12 days after laying; determined by embryonic staging), eggs were randomly assigned to either normoxic (21% O₂, partial pressure 152 mmHg) or hypoxic (10% O₂, partial pressure 72 mmHg) cohorts. The experimental gas conditions were maintained in 76-l Ziplock® bags that were connected to gas supply of either normoxia or 10% CO₂ in a sealed plastic box, containing cotton gauze soaked in isoflurane (Isoflo®, Abbott Laboratories, North Chicago, IL, United States). Once the turtles were fully anesthetized, they were euthanized by cranial and spinal pithing. Hearts were removed and weighed, and the ventricle was removed and cleared of connective tissue. The ventricle was then rinsed with ice-cold homogenization buffer (Table 1) and minced into small pieces with a razor blade. The tissue was resuspended in ice-cold homogenization buffer to remove any blood, and the suspension was homogenized in a 12-ml glass mortar using four passes of a loose-fitting Teflon pestle at 120 rpm. The homogenate was centrifuged in polycarbonate tubes at 600 g for 10 min at 4°C. The supernatant was filtered through gauze and centrifuged again at 8,500 g for 10 min. The pellet was then resuspended in fresh homogenization buffer and centrifuged again at 8,500 g for 10 min. Finally, the pellet was resuspended in ~200 µl of fresh buffer and immediately analyzed for protein content with a Bradford assay, according to the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA, United States). The mitochondrial suspension was kept on ice until assayed.

Mitochondrial Respiration and H₂O₂ Production

Respiration and H₂O₂ production were measured simultaneously with an Oxygraph O₂-k high-resolution respirometry system (Oroboros Instruments GmbH, Innsbruck, Austria), fitted with an O₂-k-fluorescence LED2-module. Two identical respiration chambers (chamber A and chamber B) were held at the same temperature (25°C) and run in parallel for each experiment. O₂ electrodes were calibrated every morning with air-saturated respiration medium (Table 1). To measure H₂O₂ production, 10 µM Amplex® UltraRed and 1 U ml⁻¹ horseradish peroxidase (HRP) were added to each chamber. Amplex® UltraRed oxidizes in the presence of H₂O₂ and forms resorufin, using HRP as a catalyst. Amplex® UltraRed was excited at 563 nm and emission was read at 587 nm; 5 U ml⁻¹ superoxide dismutase (SOD) was also added to the chambers to convert any extramitochondrial superoxide (O₂⁻) to H₂O₂. At the beginning of each experiment, isolated mitochondria (9.7–29.4 µg protein ml⁻¹) were added to each chamber containing 2 ml of respiration medium, and the Amplex UltraRed signals were calibrated with known quantities of exogenously added H₂O₂.

### Table 1 | Composition of solutions used for mitochondrial isolation and measuring respiration.

| Component                  | Homogenization buffer | Respiration medium |
|----------------------------|-----------------------|--------------------|
| Sucrose, mmol l⁻¹          | 250                   | 110                |
| HEPES, mmol l⁻¹            | 10                    | 20                 |
| EGTA, mmol l⁻¹             | 1                     | 0.5                |
| MgCl₂, mmol l⁻¹            | 1.4                   | 1.4                |
| KH₂PO₄, mmol l⁻¹           | 10                    | 10                 |
| K-MES, mmol l⁻¹            | 100                   | 60                 |
| Taurine, mmol l⁻¹          | 20                    | 20                 |
| BSA (fatty-acid-free)      | 1%                    | 1%                 |
| pH                         | 7.4                   | 7.1                |
| Preparation temperature (°C)| 4                     | Room               |

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States). pH was adjusted with KOH (5 N).
Determining Maximum Respiration Rates and H$_2$O$_2$ Production Through Individual ETC Complexes

We were interested in measuring three main variables commonly used to assess mitochondrial function. First, Leak respiration rate (Leak) is the amount of O$_2$ used to compensate for proton Leak across the mitochondrial inner membrane. Proton Leak can account for up 20–25% of routine metabolic rate in vertebrates, so a reduction in this parameter is a substantial energy-saving mechanism. Second, we measured OXPHOS respiration rate, which is the maximum rate of ADP-stimulated respiration and is an estimate of mitochondrial ATP production. Lastly, we used a protonophore, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), to uncouple mitochondrial respiration and measure the maximal respiration rate of the electron transport chain, otherwise known as state-IV capacity. We measured these three parameters with different substrate combinations, using a standard substrate inhibitor titration protocol (SUIT protocol), designed according to Pesta and Gnaiger (2012).

An original trace of the SUIT protocol is presented in Figure 1. First, pyruvate (5 mM), malate (2 mM), and glutamate (10 mM) were added to achieve Leak respiratory state with complex-I (CI) substrates in the absence of adenylates (Leak$_{N,CI}$). When O$_2$ consumption was stable, saturating ADP (5 mM) was injected to activate oxidative phosphorylation with CI substrates (OXPHOS$_{CI}$). Once ADP had been completely phosphorylated to ATP, isolated mitochondria enter the Leak respiratory state in the presence of adenylates with CI substrates (Leak$_{T,CI}$), otherwise known as state-IV respiration. Succinate (10 mM) was then added to assess the additive effects of complex-II (CII) substrates (Leak$_{T,CI+CII}$), and ADP was added again to assess OXPHOS with CI and CII substrates (OXPHOS$_{CI+CII}$).

To uncouple mitochondria and assess ET with CI and CII substrates (ET$_{CI+CII}$), FCCP was titrated to a final concentration of 0.1–0.3 µM. Next, the CI inhibitor rotenone (0.5 µM) was added to assess ET$_{CII}$, with CII substrates only. To block the electron transport chain and assess residual non-mitochondrial O$_2$ consumption (ROX), the complex-III (CIII) inhibitor antimycin-A (2.5 µM) was added. To assess complex-IV (CIV) activity in isolation, the electron donor N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD; 0.5 mM) was added in combination with ascorbate (2 mM) to avoid autooxidation of TMPD. Lastly, the CIV inhibitor sodium azide (50 mM) was added to assess background non-mitochondrial O$_2$ consumption from the addition of TMPD.

**ROS Production via Reverse Electron Transport**

Juvenile and adult turtles endure extensive periods of anoxia (up to 4 months) when they overwinter and engage in breath-hold dives (Jackson, 2002). In mammals, one of the main problems leading to anoxic cell death is a burst of mitochondrial ROS from complex I during reoxygenation, driven by the accumulation of succinate (Chouchani et al., 2016). Interestingly, recent work has shown that adult turtles limit oxidative damage at reoxygenation in the heart, by inhibiting the maximal capacity for mitochondrial ROS production via reverse electron transport (RET), in addition to limiting the accumulation of succinate (Bundgaard et al., 2019). Therefore, we reasoned that aspects of the RET pathway could be programmed by hypoxia during development, leading to improved cardiac anoxia tolerance in adulthood. To investigate this possibility, we measured H$_2$O$_2$ production in mitochondria that were incubated with succinate alone. Under these conditions, electrons are transported to the ubiquinone pool via CII and subsequently travel in reverse to CI, leading to the production of H$_2$O$_2$.

**Mitochondrial O$_2$ Affinity**

Mitochondrial O$_2$ affinity was investigated by measuring O$_2$ consumption during the transition into anoxia. Briefly, O$_2$ concentration in the chambers was reduced to ∼20% of air saturation by blowing nitrogen over the respiration media. The chambers were then closed, the mitochondria were added, and malate, pyruvate, glutamate, and succinate were injected. Saturating levels of ADP were then added and mitochondria subsequently consumed all the remaining O$_2$, thus, entering into anoxia,
while remaining in the OXPHOS state. To measure mitochondrial O₂ binding affinity, the partial pressure of oxygen where mitochondrial respiration is half maximal (P₅₀) was calculated with Prism software (v9, GraphPad, San Diego, CA, United States), using the equation

\[ P_\text{O2} = J_{\text{Max}} \cdot [O_2]/(P_{50} + [O_2]) \]

where \( J_{\text{Max}} \) is the normalized O₂-consumption rate, and \( [O_2] \) is the concentration of O₂ in the chamber.

Data Analysis

Respiration rate and H₂O₂ production were normalized to total mitochondrial protein content (Bradford assay, VersaMax spectrophotometer: Molecular Devices, Sunnyvale, CA, United States), and H₂O₂ production was also expressed relative to the rate of O₂ consumption. To estimate mitochondrial efficiency, the respiratory-control ratio (RCR) was calculated as OXPHOS CI/LeakT,CI, and the OXPHOS coupling-efficiency ratio was calculated as 1-LeakT,CI/OXPHOS CI. The OXPHOS control ratio was calculated as OXPHOS CI+CI+ETci+CI+. We also calculated the ratio of phosphorylated ADP to the atoms of consumed O₂ (P:O ratio), by making linear extrapolations of O₂ concentration during OXPHOS (immediately after ADP addition) and Leak (immediately after ADP depletion), as described by Illingworth; the difference in O₂ concentration at the intercepts is the total O₂ uptake. Statistical significances were determined using generalized linear models (GLMs), with sequential Sidak post hoc tests, for pairwise comparisons. Developmental O₂ was the between-group factor, and the pooled mitochondrial samples were the random factors. Data were considered significant when \( P \leq 0.05 \).

RESULTS

Cardiac Biometry

Exposure to hypoxia during embryonic development decreased body mass in juvenile turtles, while heart mass was unaffected, leading to a larger heart-to-body-mass ratio (Table 2).

Mitochondrial O₂ Consumption and Basal H₂O₂ Production

Mitochondrial preparations were of good quality, as attested by high RCRs (21.5 ± 1.4) and OXPHOS coupling-efficiency ratios (0.95 ± 0.003), with CI substrates (malate, pyruvate, and glutamate). Furthermore, mitochondrial oxygen consumption and H₂O₂ production responded to substrates and inhibitors in the expected manner (Pesta and Gnaiger, 2012; Figure 1).

Compared to their normoxic counterparts, juvenile snapping turtles previously exposed to developmental hypoxia had significantly lower levels of mitochondrial O₂ consumption, under all respiratory states and substrate combinations (Figures 2A–H). Basal levels of H₂O₂ production were also significantly lower in turtles exposed to developmental hypoxia (Figure 3), but this effect was only statistically significant in two respiratory states: LeakT and ET states, with CI and CII substrates (Figures 3C,F). These latter effects disappeared when H₂O₂ production was normalized to O₂ consumption (Figure 4), suggesting that the lower rates of ROS production in turtles from hypoxic incubations are due to a reduced respiratory rate. We also investigated mitochondrial capacity for H₂O₂ production with RET in the presence of succinate alone. Under these conditions, mitochondria from turtles that developed in hypoxia also had significantly lower H₂O₂ production (Figure 3I), and this effect disappeared when normalized to O₂ consumption (Figure 4).

Mitochondrial Efficiency of ATP Production and O₂ Affinity

Mitochondria from turtles exposed to developmental hypoxia were significantly more coupled (H10 RCRs were 20% higher than N21) and produced more ATP per molecule of O₂ (H10 P:O ratios were 30% higher than N21) (Figures 5A,B), indicative of an improved efficiency of ATP production. However, developmental hypoxia had no effect on mitochondrial O₂ affinity (no change in P₅₀) (Figure 5C).

DISCUSSION

Mitochondria are capable of remarkable phenotypic plasticity in response to hypoxic stress (Galli and Richards, 2014; Murray and Horscroft, 2016; Fuhrmann and Brune, 2017; Sokolova, 2018; Bundgaard et al., 2020). While this kind of remodeling is generally reversible during adulthood, fetal hypoxia can cause permanent mitochondrial abnormalities in mammals, leading to disease susceptibility (Gyllenhammer et al., 2020). Nevertheless, very little is known about the effects of developmental hypoxia on species that routinely experience O₂ deprivation during embryonic development, such as reptiles. By studying these animals, we can start to identify adaptive vs. pathological responses to developmental hypoxia. In contrast to mammals, here we show that developmental hypoxia can increase the efficiency of ATP production in juvenile snapping turtles, and lower basal ROS production. Interestingly, these responses are very similar to those observed in adult vertebrates that acclimatize to hypoxia (Johnston and Bernard, 1982; Donohoe and Boutillier, 1998; St-Pierre et al., 2000a; Heather et al., 2012; Hickey et al., 2012; Murray and Horscroft, 2016). Therefore, we speculate that developmental
hypoxia primes turtle mitochondrial physiology for hypoxic environments in adulthood.

**Developmental Hypoxia Reduces Mitochondrial Aerobic Capacity and ROS Production**

Compared to their normoxic counterparts, juvenile turtles that developed under hypoxic conditions had significantly lower rates of mitochondrial respiration. A reduction in aerobic capacity is a common response to chronic hypoxia in a wide range of adult vertebrates (Johnston and Bernard, 1982; Donohoe and Boutilier, 1998; St-Pierre and Boutilier, 2001; Heather et al., 2012; Hickey et al., 2012), including humans acclimatizing to high altitude (Murray and Horscroft, 2016) and animals that are genetically adapted to hypoxia (Ali et al., 2012; Horscroft et al., 2017). While it might seem counterintuitive to reduce aerobic capacity when oxygen is limiting, this strategy is believed to limit ROS production (Murray, 2009; Murray and Horscroft, 2016). ROS production is a major problem in hypoxia, because electrons slip from the chain more often when O2 is limited and the chain becomes more reduced (Brand, 2016). Therefore, a lower level of oxidative phosphorylation will reduce the amount of electrons in the electron transport

FIGURE 2 | Effect of developmental hypoxia on mitochondrial O2 consumption. Mitochondrial O2 consumption was measured in juvenile snapping turtles from normoxic (N21, red circles, n = 8) and hypoxic (H10, blue squares, n = 8) incubations. Each panel represents a respiratory state. (A) Leak respiration with substrates for complex I, in the absence of adenylates (LeakCI). (B) Leak respiration with substrates for complex I, in the presence of adenylates (LeakCI+ADP). (C) Leak respiration with substrates for complexes I and II, in the presence of adenylates (LeakCI+ADP+CI). (D) Oxidative phosphorylation, with substrates for complex I (OXPHOSCI). (E) Oxidative phosphorylation with substrates for complexes I and II (OXPHOSCI+CI). (F) Electron-transfer capacity, with substrates for complexes I and II (ETCI+CI). (G) Electron-transfer capacity, with substrates for complex II (ETCI). (H) Electron donation to complex IV (CV). Statistical significance was assessed with individual generalized linear models, followed by Sidak post hoc tests, to assess the effect of developmental O2 (N21 vs. H10). Values were considered significant when P ≤ 0.05, which are denoted by asterisks (*).
FIGURE 3 | Effect of developmental hypoxia on mitochondrial $\text{H}_2\text{O}_2$ production. Mitochondrial $\text{H}_2\text{O}_2$ production was measured in juvenile snapping turtles from normoxic (N21, red circles, $n = 6$) and hypoxic H10 (blue squares, $n = 7$) incubation. Each panel represents a respiratory state. (A) Leak respiration with substrates for complex I, in the absence of adenylates ($\text{Leak}_{\text{N1}}$). (B) Leak respiration with substrates for complex I, in the presence of adenylates ($\text{Leak}_{\text{T1}}$). (C) Leak respiration with substrates for complexes I and II, in the presence of adenylates ($\text{Leak}_{\text{T1+T2}}$). (D) Oxidative phosphorylation, with substrates for complex I ($\text{OXPHOS}_{\text{N1}}$). (E) Oxidative phosphorylation, with substrates for complexes I and II ($\text{OXPHOS}_{\text{N1+N2}}$). (F) Oxidative phosphorylation, with substrates for complex I ($\text{OXPHOS}_{\text{T1}}$). (G) Electron-transfer capacity, with substrates for complex II ($\text{ET}_{\text{N2}}$). (H) Electron-transfer capacity, with substrates for complex II and antimycin-A ($\text{ET}_{\text{T2+a}}$). (I) Electron-transfer capacity, with substrates for complex II and ATP synthase inhibitors ($\text{ET}_{\text{T2+c2}}$). Statistical significance was assessed with individual generalized linear models, followed by Sidak post hoc tests, to assess the effect of developmental $\text{O}_2$ (N21 vs. H10). Values were considered significant when $P \leq 0.05$, which are denoted by asterisks (*).
Although it was not a focus of this study, our experimental design gives some insight into the mechanisms reducing respiratory rate in turtles from hypoxic incubations. Given that the reduction was observed under all respiratory states and substrate combinations, the underlying mechanism is likely to be something fundamental to the entire electron transport chain (Brand and Nicholls, 2011). One possibility is that developmental hypoxia reduced the volume or cristae density of turtle cardiac mitochondria. Indeed, a downregulation of mitochondrial biogenesis is a common response found in adult vertebrates exposed to chronic hypoxia at high altitude (Murray, 2009). Additionally, a widescale reduction in respiration rates can also occur via a reduction in the expression or activity of complex IV (cytochrome c oxidase), which would limit the respiratory capacity of the entire chain. However, this modification would also increase the reduction state of the electron transport chain, leading to higher rates of ROS production (Dawson et al., 1993). Lastly, several studies have shown that environmental stress during development can epigenetically program mammalian antioxidant-defense systems, which could theoretically alter basal levels of ROS production in adulthood (Strakovsky and Pan, 2012). In this respect, it is interesting to note that hypoxia tolerance is closely related to antioxidant capacity in ectothermic vertebrates (Bickler and Buck, 2007), including turtles (Storey, 1996).
the mitochondrial membrane without the generation of ATP phenomenon refers to the process whereby protons leak across that accounts for individual variation is proton leak. This can influence the P:O ratio, the most common mechanism 2004; Stier et al., 2014; Salin et al., 2015). While several factors including growth, reproduction, and lifespan (Barros et al., 2013; Zhang et al., 2013; Murray and Horscroft, 2016). Interestingly, developmental hypoxia has been

FIGURE 5 | Mitochondrial efficiency of ATP production and O2 affinity in juvenile snapping turtles from normoxic (21% O2; N21) and hypoxic (10% O2; H10) incubations. (A) P:O ratio. Oxygen concentration as a function of time is shown for normoxic (n = 5) and hypoxic (n = 5) developmental groups. Solid lines correspond to the line of best-fit. The inset graph shows the mean P:O values ± SEM. (B) The respiratory-control ratio (RCR) and (C) OXPHOS coupling efficiency ratio were calculated for normoxic (n = 5–8) and hypoxic (n = 5–8) developmental groups. Both ratios were measured in the presence of complex-I substrates. (D) Mitochondrial oxygen affinity. Respiration rate (R2O2) as a function of oxygen concentration is shown, with individual curves plotted for normoxic (n = 5) and hypoxic (n = 5) incubation. The inset graph shows the mean R2O2 values ± SEM. Calculations for all variables are given in Section "Materials and Methods." Statistical significance was assessed with individual generalized linear models, followed by Sidak post hoc tests, to assess the effect of developmental O2 (N21 vs. H10). Values were considered significant when P ≤ 0.05, which are denoted by asterisks (*).

Clearly, further experiments are necessary to confirm the mechanism that program respiratory rate and ROS production in turtles from hypoxic incubations. Future research should be directed toward investigating the effects of developmental hypoxia on turtle mitochondrial morphology with electron microscopy, as well as the expression or activity of electron-transport-chain complexes and antioxidants. Furthermore, it would be interesting to measure the activity of anaerobic enzymes, such as lactate dehydrogenase, which are known to be altered by developmental hypoxia in some ectotherms (Matschak et al., 1998; Ton et al., 2003; Anderson and Podrabsky, 2014).

Developmental Hypoxia Increases the Efficiency of Mitochondrial ATP Production

In order to offset the reduced aerobic capacity that accompanies chronic hypoxia, many adult animals increase the efficiency of mitochondrial ATP production by reducing proton leak and increasing the P:O ratio (Gnaiger et al., 2000; Semenza, 2007; Zhang et al., 2013; Murray and Horscroft, 2016). Interestingly, we found that developmental hypoxia programmed these same attributes in turtle ventricular mitochondria. The P:O ratio is a measure of the number of ATP molecules synthesized by oxidative phosphorylation for each oxygen atom reduced. Although oxygen consumption is routinely used as a proxy for ATP production, the amount of ATP generated per unit of oxygen consumed can vary significantly between individuals, and it is well established that environmental stress can alter this parameter (Salin et al., 2015). Improvements in the P:O ratio can have major implications for animal life-history traits, including growth, reproduction, and lifespan (Barros et al., 2004; Stier et al., 2014; Salin et al., 2015). While several factors can influence the P:O ratio, the most common mechanism that accounts for individual variation is proton leak. This phenomenon refers to the process whereby protons leak across the mitochondrial membrane without the generation of ATP (Divakaruni and Brand, 2011). Importantly, proton leak can account for up to 25% of an animal’s metabolic rate, so a reduction in this parameter can greatly increase the efficiency of ATP production. Therefore, the elevation in the P:O ratio we observed in turtles from hypoxic incubations could be driven by a decrease in proton leak. In support of this contention, we found that developmental hypoxia significantly lowered levels of leak respiration, leading to an increase in mitochondrial coupling (higher RCR values). Interestingly, we found the same result in juvenile alligators exposed to developmental hypoxia (Galli et al., 2016), suggesting that proton-leak pathways are a common target for developmental programming. Future research should, therefore, determine the mechanism driving the reduction in proton leak, which could include changes to the inner mitochondrial membrane, like the composition of the lipid bilayer, expression of the adenine nucleotide translocase, and/or suppression of uncoupling proteins (Zhao et al., 2019). Nevertheless, it should be noted that a decrease in proton leak in the absence of other changes will lead to an increase in ROS (Brand, 2000).

Developmental Hypoxia Has No Effect on Mitochondrial Oxygen Affinity

Several studies have shown that mitochondrial P50 is lower in hypoxia-tolerant vs. hypoxia-sensitive species (Scott et al., 2011; Zhang et al., 2013; Lau et al., 2017), and it can be decreased in some adult vertebrates after acute and chronic episodes of hypoxia (Gnaiger et al., 1998b; St-Pierre et al., 2000b). A low P50 allows the mitochondria to extract oxygen from the cytosol more effectively, which is particularly important during periods of hypoxia, when the concentration gradient for O2 has been reduced (Gnaiger, 2001). Several mechanisms can account for these differences, including changes in the abundance and activity of complex IV (the site of oxygen reduction), altered flux through the electron transport chain relative to complex-IV capacity,
and differences in the mitochondrial membrane potential (Bienfait et al., 1975; Verkhovsky et al., 1996; Gnaiger et al., 1998a). Nevertheless, in the present study, we did not find any differences in mitochondrial O$_2$-binding affinity between the developmental cohorts.

**Physiological Significance and Perspectives**

Adult vertebrates acclimatizing to hypoxic environments undergo mitochondrial remodeling to enhance oxygen delivery, maintain ATP balance, and limit oxidative stress. The mechanisms underlying this remodeling include a reduction in aerobic capacity and proton leak, a reduced basal ROS production, and an increase in the P:O ratio. For the first time, we show that these same attributes can be permanently programmed in turtles by exposure to hypoxia during embryonic development. The molecular mechanisms underlying this response remain to be investigated, but they could include hypoxia-inducible factor (HIF) signaling. Indeed, HIF-1 activation is known to coordinate mitochondrial remodeling in adult vertebrates exposed to hypoxia (Murray and Horscroft, 2016; Thomas and Ashcroft, 2019), and this pathway is activated in hypoxic zebrafish embryos, leading to enhanced hypoxia tolerance in adulthood (Robertson et al., 2014). In addition, mammalian mitochondria can be programmed during development via epigenetic alterations to the nuclear and mitochondrial genomes, changes in the mitochondrial–telomere axis, and the accumulation of mitochondrial DNA defects (Gyllenhammer et al., 2020). Whatever the mechanism, we suspect that mitochondrial programming in turtle embryos will improve hypoxia tolerance in adulthood, which would be beneficial for breath-hold diving and overwintering in anoxic environments (Jackson, 2000). Indeed, our recent study suggests turtles from hypoxic incubations have improved cardiac anoxia tolerance (Ruhr et al., 2019). Therefore, developmental hypoxia could represent an important environmental cue for turtles that primes their physiology for a future in low-oxygen environments.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

The animal studies were carried out according to the approved animal care protocol of the University of North Texas Institutional Animal Care and Use Committee No. 1403-04.

**AUTHOR CONTRIBUTIONS**

IR and GG: conceptualization, methodology, formal analysis, and writing—original draft. IR, GG, JC, and DC: investigation. GG and DC: resources, supervision, and project administration. IR, GG, and DC: writing—review and editing and funding acquisition. IR: visualization. All authors contributed to the article and approved the submitted version.

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