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The effects of low dissolved oxygen concentrations on diving behavior and lactate accumulation in *Lithobates pipiens*

ABSTRACT

Dissolved oxygen (DO) concentrations in bodies of water can differ based on geographic location, time of day, or even climate change. Because frogs acquire approximately 20% of their oxygen across the skin, decreases in DO could negatively affect diving behavior by increasing reliance on anaerobic metabolism and formation of lactate. To evaluate this possibility, we measured pulmonary and cutaneous oxygen exchange as well as blood lactate levels before and after a 30-minute dive period under both saturated DO (8.341 ± 0.042 mg/L) and low DO conditions (3.918 ± 0.597 mg/L). Although frogs diving in a low oxygen environment tended to accumulate slightly more lactate, which would indicate a shift toward anaerobic metabolism, the trend was not significant ($t(8) = 1.86$, $p = 0.525$). A reduction in cutaneous O₂ gas exchange was observed to be significant ($t(8) = 1.86$, $p = 0.0015$) following a forced dive. However, cutaneous O₂ gas exchange only makes up a miniscule amount of the total pulmocutaneous gas exchange; therefore, would have little effect on lactate accumulation. In conclusion, low DO water does not appear to influence diving behavior or lactate accumulation in a biologically significant way for this species. However, we acknowledge the possibility that low DO water may have a more profound effect during dives of longer durations.

INTRODUCTION

The amount of dissolved oxygen in water is dependent on multiple factors such as temperature, atmospheric (atm) pressure, and relative concentration of other dissolved substances. There are concerns regarding dissolved oxygen in Earth's bodies of water due to climate change; given that extreme reductions in carbon emissions are needed to simply stall changes in atmospheric radiative properties (Matear, 2003), it has been estimated that dissolved oxygen in the ocean will continue to decrease by 4-7% in the coming century (Bopp et al, 2002). Cooler temperatures, lower altitude, and water with a low relative concentration of dissolved substances will contain higher levels of dissolved oxygen than their individual counterparts (Risberg, 2018).

The aforementioned variables can also have an impact on physiological diving mechanics of frogs, including *L. pipiens*, in addition to their effects on dissolved oxygen concentration. Diving patterns, such as aerobic dive limit (ADL), can vary based on time of day, temperature, or breeding cycles. A frog's ADL is the amount of time they may spend using aerobic metabolism while submerged before needing a switch to anaerobic metabolism. Environmental conditions that decrease dissolved oxygen in water will have a negative impact on a frog's ADL (Butler, 2006). Northern Leopard Frogs typically do not move very far, but move both during the day and night (Merrell, 1977). This species relocates between winter and breeding locations, usually in

April, then back to winter sites around August (Merrell, 1977). Studies performed also depict seasonal changes in temperature and atmospheric pressure affect heart rate; for example, winter conditions have been shown to produce bradycardia and arrhythmia in *Rana temporaria* (Rocha and Branco, 1998).

The path of blood flow in frog hearts begins with oxygen-poor blood entering the right atrium and oxygen-rich blood entering the left atrium. Since frogs only have one ventricle, the blood from both the systemic and circulation and the pulmonary circulation mix once they enter the third chamber. The spiral valve, however, divides the pool of blood based on oxygen content; oxygen-poor blood is diverted to the pulmocutaneous circuit, and oxygen-rich blood goes to the systemic circuit (Boutilier et al., 1986). The pulmocutaneous circuit is able to direct the flow of oxygen-poor blood to both the lungs or the surface of the skin. This is controlled by muscular sphincters that contract either the cutaneous artery or the pulmonary artery resulting in reduced blood flow for the circuit it leads to; blood will then flow to the circuit with its sphincter relaxed (Cotton, 2016).

This system is extremely beneficial for organisms, such as frogs, that spend prolonged periods of time under water during harsh conditions. Species of frogs living in Minnesota, like *L. pipiens*, tend to spend their winters beneath ice, submerged for great periods of time. Thus, they must rely solely on cutaneous gas exchange for oxygen consumption and CO₂ expulsion. If cutaneous gas exchange does not meet oxygen demands then these frogs must use proper oxygen consumption and CO₂ expulsion while submerged to avoid or alleviate anaerobiosis and metabolic acidosis (Jackson, 2004). Frogs are unable to use their lungs and must rely solely on cutaneous CO₂ expulsion during a forced dive. While efficiency of CO₂ expulsion across the skin is upwards of 90%, submergence without ventilation leads to respiratory acidosis. While submerged, frogs use their internal sphincters to divert oxygen-poor blood to the cutaneous circuit where gas exchange can still occur at the skin surface. A thinner epidermis means a shorter profusion distance-this makes frogs with a thin epidermis more proficient at gas exchange. Additionally, by increasing the amount of capillary beds perfused, frogs can increase their skin surface area available for diffusion.

To further analyze this relationship between the cutaneous and pulmonary circuits, we were interested in the relative contribution of cutaneous and pulmonary gas exchange in both free-swimming and forced-diving frogs. We would expect an upregulation in the cutaneous circuit during a forced dive to increase O₂ consumption and CO₂ expulsion. If there is no change in gas exchange rates, an increase in anaerobic metabolism during a forced dive will create an O₂ debt. This debt can be shown through an increased lactate concentration in the blood (Boutilier and Shelton, 1986). The frog will then attempt to relieve this O₂ debt by increasing pulmonary gas exchange during the recovery dive. In free-swimming frogs, on the other hand, a voluntary dive can be ended as the frog chooses. We therefore hypothesize that voluntary dives will be terminated before the frog must turn to anaerobic metabolism, so no O₂ debt would occur.

MATERIALS AND METHODS

A total of nine observably healthy *L. pipiens* were used for this experiment. Each frog was weighed prior to testing (body weight 19-50 grams) and weights were held fairly constant throughout project with bidaily feeding of mealworms. Frogs were placed in a closed system

respirometry chamber following weight measurement. The chamber was partially filled with water and two Vernier probes were used to measure O₂ consumption (umol/min*g). A gas O₂ probe was placed within the headspace to measure pulmonary oxygen consumption while the dissolved O₂ probe was submerged in water. Water was well-mixed by a stir bar at the bottom of the container and a protective covering was placed over top to ensure no harm to the frog. Vernier software was used on a computer to collect experimental data throughout each run and for later data analysis (Cotton, 2016).

During the first portion of trials, DO concentrations were altered by bubbling nitrogen into the water. Nitrogen (N₂) bubbling has been shown to be the most effective means of decreasing oxygen concentrations in water (Butler, 1994). While use of this method did prove to be the most efficient, it also caused nitrogen narcosis symptoms in the frogs. Therefore, alternative methods were sought to decrease DO concentration while also being safe for the frogs to ensure trial completion. According to Butler et al., there are four common methods for removal of dissolved oxygen: boiling at 1 atm, boiling under reduced pressure, sonication under reduced pressure, and bubbling of N₂ (Butler et al., 1994). While boiling at 1 atm was shown to be least effective in DO removal, this process was used due to equipment availability and relative ease. Water was boiled in an erlenmeyer flask for approximately 30 minutes before taken off the hotplate and a rubber stopper placed in the top. The flask was left overnight to be used for experimental run the following day.

The experiment for each frog was split up into three distinct runs: a voluntary, forced, and recovery dive. Setup for both the control and recovery dives consisted of filling the 1.25L reservoir with 0.92L of water, leaving 0.33L of air. These conditions allowed for both pulmonary and cutaneous oxygen exchange. The reservoir was filled completely with water for the forced dive run, forcing the frogs to only use pulmocutaneous gas exchange. Manipulation of DO concentration between high DO concentration (8.341 ± 0.042 mg/L) and low DO concentration (3.918 ± 0.597 mg/L) was done during the forced dive. Our independent variable was the relative DO₂ concentration during the forced dive run. Our dependent variable was time and relative concentration of DO₂ during the control and recovery dives. An oxygen bubbler was placed in the water reservoir 1 hour before each experiment to increase DO₂ concentrations. Each run lasted 30 minutes in which total amount of time spent diving was observed and recorded. Following each 30 minute run, blood samples were taken to measure lactate concentration for later analysis. To minimize risk of infection, the area of sampling was previously sanitized with antiseptic Bactine and wiped dry. Blood samples were obtained by quick insertion and withdrawal of a small hypodermic needle into the facial vein, just anterior to the tympanum. A capillary tube was used to collect blood pooling on the skin surface (Forzán et al., 2012). Lactate concentrations were measured using a lactate meter (Nova Biomedical; Waltham, MA). Hemostasis was achieved with a small amount of pressure over the puncture site. This procedure was repeated until three distinct runs were completed twice for each frog. Each frog participated in the two trials consisting of three separate 30-minute runs.

RESULTS

Upon observation of dissolved oxygen concentration effects during a forced dive, results showed a significant reduction in total pulmocutaneous gas exchange. But, reduction of dissolved oxygen concentrations was more effective on total pulmocutaneous gas exchange during forced dive (See Figure 1).

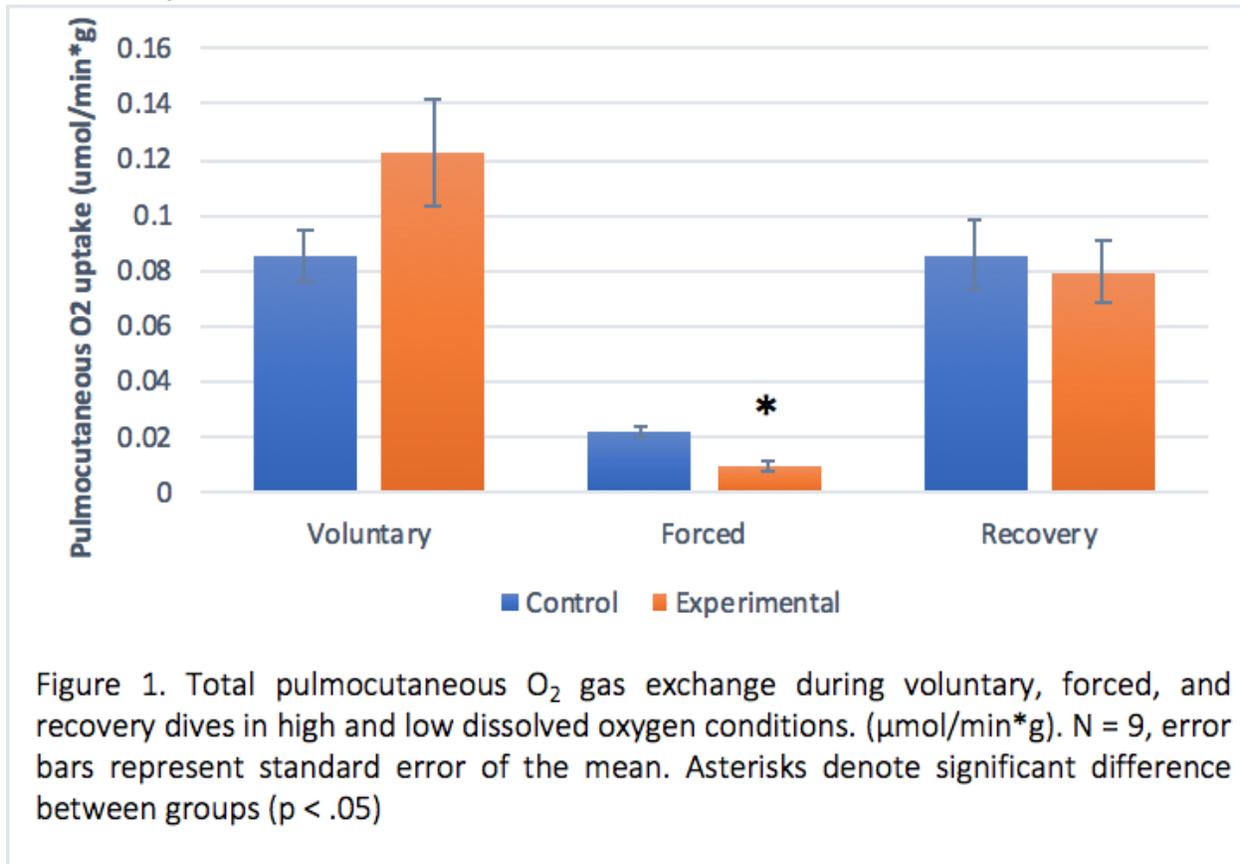


Figure 1. Total pulmocutaneous O₂ gas exchange during voluntary, forced, and recovery dives in high and low dissolved oxygen conditions. (μmol/min*g). N = 9, error bars represent standard error of the mean. Asterisks denote significant difference between groups (p < .05)

Figure 1 shows the total pulmocutaneous gas exchange rates during voluntary, forced, and recovery dives. There was no significant difference between low and high DO groups during the voluntary dive ($p > .05$) allowing these groups to be compared. Between a voluntary and forced dive, there was a significant reduction in total pulmocutaneous gas exchange for both low and high DO concentrations ($p < .05$). Total pulmocutaneous gas exchange during a forced dive solely reflects cutaneous gas exchange due to apnoea. Rates of total pulmocutaneous gas exchange for low and high DO concentrations were significantly different following a forced dive ($p < .05$). Total pulmocutaneous gas exchange was increased between forced and recovery dives. During the recovery dive, rates of total pulmocutaneous gas exchange were not significantly different between the two groups. While insignificant, total pulmocutaneous gas exchange following the recovery dive in a low DO environment was lower than that of the control group (see Table 1). A reduction in total pulmocutaneous gas exchange during a forced dive leads to increases in blood lactate accumulation (see Figure 2).

There were no differences in pulmonary oxygen uptake between low and high dissolved oxygen groups during voluntary and recovery dives. Rates of cutaneous O₂ uptake doubled for

the control group during a forced dive while there was no change in the experimental group (see Table 1).

Table 1. Relative use of pulmonary and cutaneous gas exchange during voluntary, forced, and recovery dive with high (control) and low (experimental) dissolved oxygen concentration ($\mu\text{mol O}_2/\text{min}\cdot\text{g}$).

Pulmonary O₂ Uptake		
($\mu\text{mol} / \text{min} \cdot \text{g}$)	Control	Experimental
<i>Voluntary Dive</i>	0.075 ± 0.01	0.114 ± 0.02
<i>Forced Dive</i>	NA	NA
<i>Recovery Dive</i>	0.075 ± 0.01	0.071 ± 0.01
Cutaneous O₂ Uptake		
($\mu\text{mol} / \text{min} \cdot \text{g}$)	Control	Experimental
<i>Voluntary Dive</i>	0.011 ± 0.0021	0.009 ± 0.0004
<i>*Forced Dive</i>	0.022 ± 0.002	0.009 ± 0.002
<i>Recovery Dive</i>	0.011 ± 0.0016	0.009 ± 0.0007

*Indicates significant differences between control and experimental groups ($p < 0.05$).

Forced dives increased lactate concentrations in both experimental and control groups. However, decreasing the concentration of dissolved oxygen had no effect on the lactate accumulation, although there was a trend for frogs in the low dissolved oxygen group to have higher lactate concentrations following forced and recovery dives (see Figure 2).

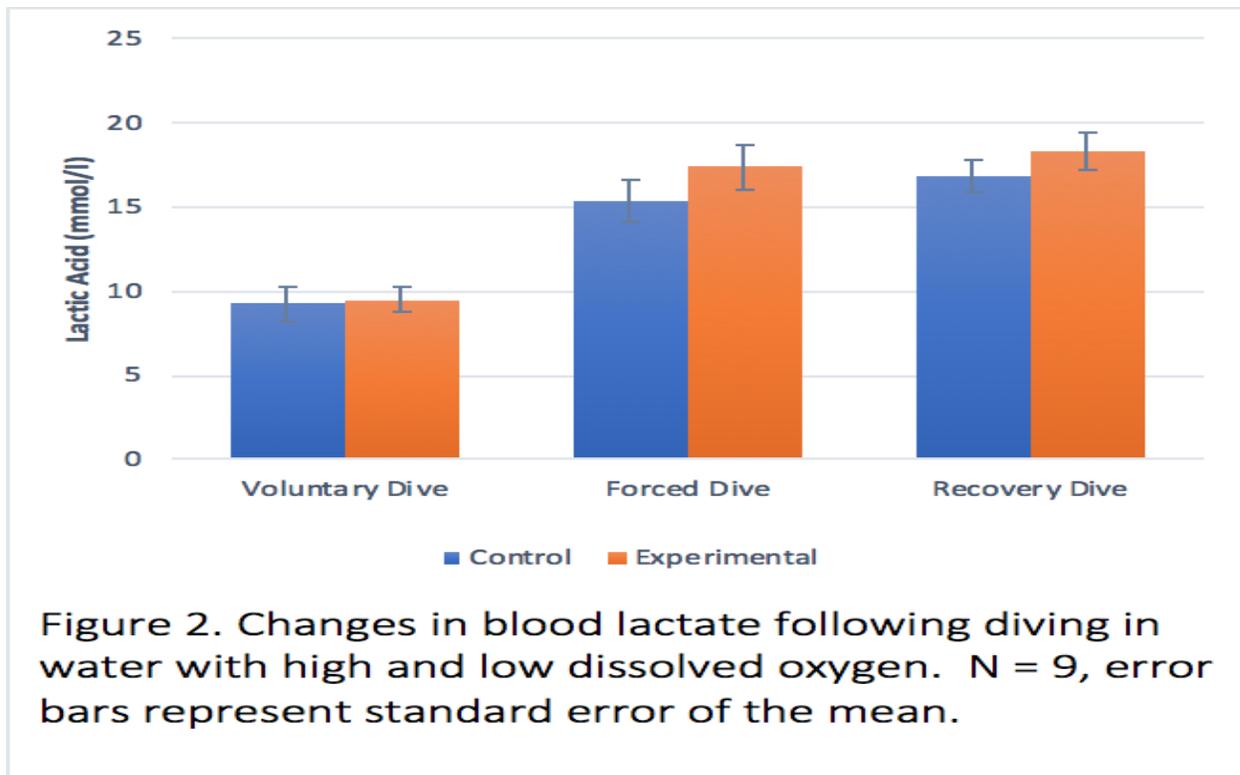


Figure 2 shows the blood lactate levels between low and high DO groups for each of the three dives. Blood lactate accumulation increased between voluntary, forced, and recovery dives for both groups. The increase in lactate accumulation was significant for both groups between voluntary and forced dives ($p < .05$), but not between forced and recovery dives.. There was no significant difference between low and high DO groups for any of the dive types, yet there was a trend increase after each dive.

Results show that variations to dissolved oxygen concentration have a significant impact on total pulmocutaneous gas exchange rates during a forced dive, but no significant impact on blood lactate accumulation.

DISCUSSION

For our experiment, we hypothesized that reduction of dissolved oxygen concentration would lead to lower rates of cutaneous gas exchange and higher blood lactate levels due to increased reliance on anaerobic pathways. Rates of gas exchange were significantly reduced during the forced dive for both groups, showing the small amount of O_2 consumption performed by the skin. Reducing the relative dissolved oxygen concentration had a significant impact on the rate of cutaneous gas exchange for the low dissolved oxygen group (Table 1). This reduction in cutaneous gas exchange for low dissolved oxygen group would increase the reliance on anaerobic pathways for energy. However, since O_2 consumption via cutaneous uptake makes up such a small portion of total gas exchange, a reduced rate would not have any significant impact on blood lactate accumulation (Figure 2).

While in a forced dive frogs are unable to ventilate their lungs and must rely on cutaneous gas exchange to meet their metabolic needs. Decreasing the relative concentration of dissolved

oxygen in the water makes it harder for frogs to meet aerobic metabolic demands, hence an increased reliance on anaerobic pathways. The ability of frogs to consume oxygen via cutaneous uptake is dependent on the diffusive capacity of the O₂ gradient. Diffusion capacity may be regulated through recruitment of underperfused capillaries in the skin during low dissolved oxygen conditions. Recruitment of underperfused capillaries increases the surface area available for cutaneous O₂ consumption and CO₂ expulsion. This recruitment of underperfused capillaries allows the frog to uptake more oxygen from the environment, which is important during hypoxic conditions in battling buildup of metabolic lactic acid (Tattersall et al., 2012). Vasodilation of cutaneous blood vessels also increases diffusion capacity of the skin in combating hypoxic buildup of lactic acid via anaerobic metabolism (Donohoe and Boutilier, 1997). Rates of lactic acid buildup did increase between voluntary and forced dives (Figure 2), but they were not significant ($p > .05$). Recruitment of underperfused capillaries and vasodilation of cutaneous blood vessels may alleviate buildup of lactic acid, allowing frogs to remain submerged for longer periods of time. Frogs in the control group had rates of cutaneous gas exchange that were double of rates measured during voluntary and recovery dives. There was no change in cutaneous oxygen exchange in the low DO group despite decreasing the concentration gradient for O₂ by half (control: 8.341 ± 0.042 mg/L, experimental: 3.918 ± 0.597 mg/L). This would support increased cutaneous blood flow via increased diffusion capacity and vasodilation. While cutaneous blood flow may increase for both groups, perhaps blood flow is insufficient to compensate for the reduced gradient in the low DO group.

Increases in blood lactate accumulation were significant between a voluntary and forced dives, showing that cessation of lung ventilation leads to anaerobiosis. Decreasing the concentration of dissolved oxygen had no effect on the lactate accumulation due to the relative unimportance of cutaneous oxygen consumption compared to pulmonary oxygen consumption. The relative unimportance of cutaneous oxygen consumption is the reason by we see so little difference in blood lactate levels for each of the three runs (see Figure 2). Although insignificant, there was a general trend for lactate levels to be higher for the experimental groups in each of the three runs. Prolonged dives exceeding ADL encourages the frog to shunt blood to their CNS and skin for proper brain functioning and cutaneous gas exchange (Butler, 2006). Therefore, increasing lactate accumulation between the forced and recovery dive may be explained by reperfusion of hypoxic-tolerant tissues such as muscle (Dewey, 1999). The insignificant increase in lactate accumulation may also depend on the relative rate of removal from the blood. Lactate may be removed through urinary excretion or conversion to pyruvate (Higgins, 2007). Lactate is freely filtered by the glomerulus, but upwards of 98% is reabsorbed making urinary excretion least favorable. Conversion to pyruvate may continue down the oxidative phosphorylation pathway to generate high ATP yield in the mitochondria or be converted to glucose via gluconeogenesis in the liver (Higgins, 2007).

During periods of submergence, the lungs of *L. pipiens* can act as a source of stored O₂ (Fernandez and Glass, 2011), which may be accessed to prolong aerobic metabolism. Storage of O₂ by the lungs is suggested to be unimportant in voluntary dives (Boutilier and Shelton, 1986), because frogs are freely able to use their lungs for ventilation. Diving produces a left-to-right shunt that reduces the perfusion of the lung (Fernandez and Glass, 2011), reducing the use of their O₂ store. Using less of the lungs O₂ store will allow for a prolonged access to adequate O₂ for aerobic metabolism, decreasing reliance on anaerobic pathways. Reduced perfusion of the lung O₂ store

during hypoxic conditions may alleviate production of lactate during a forced dive (see Figure 1). During the recovery dive, rates of pulmonary ventilation were not significantly different between the two groups (see Table 1).

Periods of apnoea make it increasingly difficult for frogs to take in sufficient oxygen to meet metabolic demands. An inability to meet metabolic demands during a forced dive leads to respiratory acidosis (Boutilier and Shelton, 1986). Respiratory acidosis leads to increases in $p\text{CO}_2$ and HCO_3^- , which may be combated with increased ventilation of the lungs during the recovery dive. However, rates of pulmonary ventilation were not significantly different between the two groups during the recovery dive (see Table 1). The lowering of P_{50} in hypoxic conditions during the forced dive may be a possible alternative avenue for compensation. In a hypoxic environment, relative O_2 concentrations are lower making it increasingly difficult to uptake adequate oxygen from the environment. To maximize O_2 uptake during these hypoxic conditions, frogs will facilitate oxyhemoglobin saturation at a lower $p\text{O}_2$ by lowering P_{50} . Therefore, less oxygen is needed to get a higher blood oxyhemoglobin saturation in the blood leading to better oxygen delivery with less oxygen (Emilio, 1973).

CONCLUSION

Variations in dissolved oxygen concentrations, along with environmental factors, play a role in diving behavior and lactate accumulation in *L. pipiens*. Decreasing the relative concentration of dissolved oxygen lead to decreases in cutaneous gas exchange during a forced dive, but no changes in blood lactate accumulation. Alterations to cutaneous blood flow, diffusion capacity, lung O_2 storage, P_{50} , and lactate removal are all possible pathways for combating a hypoxic environment. Combating lactate accumulation via these pathways allows *L. pipiens* to remain submerged for longer periods of time without major consequences.

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