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Effects of Dissolved Oxygen on Northern Leopard Frog (Lithobates pipiens) Gastrocnemius Muscle Recruitment and Fatigue

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EFFECTS OF DISSOLVED OXYGEN ON NORTHERN LEOPARD FROG (*LITHOBATES PIPIENS*) GASTROCNEMIUS MUSCLE RECRUITMENT AND FATIGUE

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Abstract

Dissolved oxygen levels in aquatic systems have decreased because of temperature increases caused by climate change, which in turn has affected ecosystems and wildlife. Many physiological processes in aquatic organisms require a certain dissolved-oxygen range, and decreasing levels can compromise proper functioning. Previous studies have linked muscle performance to dissolved oxygen levels in a variety of aquatic species, but less research has been dedicated to amphibians. Because many amphibians engage in cutaneous respiration, especially when dwelling in aquatic habitats, dissolved oxygen levels may have a significant impact on muscle performance in this taxon. This experiment investigated the effects of dissolved oxygen and time in vitro on frog skeletal muscle contractile force and fatigue. Results did not vary significantly when dissolved oxygen was altered, but fatigue and contractile force experiments did correlate with time in vitro. Although we did not find an effect of dissolved oxygen levels on muscle characteristics in vitro, a better understanding of the effects of dissolved oxygen on muscle performance, particularly in vivo, could be beneficial as climate change alters the oxygen content of aquatic systems, with the potential to affect physiology and behavior.

Keywords: cutaneous respiration, dissolved oxygen, contractile force, fatigue, in vitro

Introduction

It is known that the solubility of oxygen is temperature-dependent and that bodies of water with lower temperatures will have a higher dissolved oxygen content than those of higher temperatures. Both fresh- and saltwater have recently been observed to fluctuate in dissolved oxygen, and researchers have attributed this to climate change and nutrient over-enrichment (Irby et al., 2018; Whitehead et al., 2009). Specifically, the proportional increase in stream temperature with air temperature as a result of climate change has caused decreases in dissolved oxygen

levels (Ficklin et al., 2013). Similarly, nutrient runoff from agriculture in the Mississippi basin affects downward bodies of water and ecosystems, reflected by decreased dissolved oxygen in the Gulf of Mexico (Joyce, 2000). The decreased dissolved oxygen levels caused by increased stream and air temperatures as a result of climate change can have severe effects on biodiversity, behavior, and distribution of wildlife inhabiting these bodies of water. This includes decreased growth rates of wildlife in hypoxic waters and, in some extreme hypoxic conditions, creation of aquatic dead zones that cannot sustain life (Breitburg, 2002; Joyce, 2000; Waldrop et al., 2018). With increasing concerns of the effects of increased air and water temperatures and the decrease in dissolved oxygen content in these waters on aquatic ecosystems and wildlife, further investigation of the relationship between oxygen availability and organism performance can provide insight on the consequences of decreased dissolved oxygen levels.

Dissolved oxygen can contribute to population-level variation in aquatic systems, such as reduced growth and development (Breitburg, 2002), which, mechanistically, may in part be due to the relationship between oxygen availability and the underlying physiology of an organism. Oxygen transport capacity has effects on metabolic rate, as hypoxic conditions are negatively correlated with metabolic activity in some reptiles and amphibians (Gangloff & Telemeco, 2018). This is particularly evident in the relationship between oxygen availability and muscle performance and fatigue. Muscle performance is sustained through a consistent supply of oxygen for oxidative phosphorylation and adenosine triphosphate (ATP) genesis (Wittenberg and Wittenberg, 1989). Muscle fatigue can be attributed to ATP availability, in that if muscle activity exceeds ATP synthesis rates, then fatigue is observed (Sundberg & Fitts, 2019). In hypoxic conditions, muscle fatigue has been shown to occur earlier (Amann et al., 2006; Verges et al., 2010), as decreased oxygen is directly related to ATP depletion (Sahlin et al., 1998). The effects of dissolved oxygen levels on the muscle physiology of aquatic animals are well documented (Waldrop et al., 2018). For example, aquatic species such as whiteleg shrimp (*Litopenaeus vannamei*) decrease distance traveled per tail-flip, and crucian carp (*Carassius carassius*) have shown a decrease in maintained fastest swimming speed in low-oxygen environments (Domenici et al., 2013; Duan et al., 2022; Penghan et al., 2014). Overall, these studies demonstrate the importance of dissolved oxygen levels on muscle-based performance in aquatic environments.

Much of the research on dissolved oxygen in aquatic systems and muscle performance has been conducted in fish and invertebrates, with comparatively less research devoted to the effect of dissolved oxygen concentration on muscle performance in amphibians. This is significant because many amphibians engage in some degree of cutaneous respiration, using their skin as a membrane for gas exchange. While lungs and gills (in amphibian larvae) generally provide both a thinner surface and increased surface area for gas exchange, making it preferable for gas

exchange, many amphibians can take in as much as 30% of their oxygen through the skin, particularly when metabolic rate is lower (Feder & Burggren, 1985). Additionally, some amphibian species have adaptations to increase the surface area of the skin, while other species increase capillary perfusion during high respiratory activity, such as mating rituals (Feder & Burggren, 1985). This suggests that cutaneous respiration has a functional role in oxygen uptake and may be affected by levels of dissolved oxygen, potentially affecting muscle performance as well; however, this concept has not been well studied.

Because oxygen is required for proper muscle performance, and declining dissolved oxygen may modulate this relationship, we investigated the effects of dissolved oxygen on in vitro skeletal muscle contractile force and fatigue in northern leopard frogs (*Lithobates pipiens*). We hypothesized that bathing a muscle with varying dissolved oxygen levels would affect muscle performance properties. Specifically, we predicted that decreased dissolved oxygen would decrease contractile force and decrease the time to fatigue.

Materials and Methods

Fifteen northern leopard frogs were acquired from Carolina Biological Supply (Burlington, North Carolina). This species is known to respirate cutaneously when hibernating in water (Tattersall & Boutilier, 1997). Frogs were housed in pairs in a plastic enclosure (34.5 cm x 30 cm x 13 cm) containing rocks and water. The frogs were each fed 8–10 mealworms a day and were held in captivity for up to two days. All procedures were approved by the Pennsylvania State University's Institution Animal Care and Use Committee (protocol #201546456).

The frogs were humanely sacrificed, and the gastrocnemius muscle was separated from the tibiofibular bone at the insertion point. Ringer's solution (200–250 mOsm, 6.6 g/L NaCl, 0.15 g/L KCl, 0.15 g/L $CaCl_2$, 0.2 g/L $NaHCO_3$) was used to keep the muscle moist after removal. The femur was left attached to the tibiofemoral joint, keeping it connected to the gastrocnemius at the muscle origin. A thread was tied around the interface of the muscle and Achilles tendon, with the other end of the string attached to a force transducer to record muscle force. To apply a stimulus voltage, a stimulating rod was attached to a ring stand and positioned so it was in contact with the middle of the muscle.

Both legs from each frog were used in the experiment, one as treatment and one as control. We systematically alternated which leg muscle (right or left) went into the control and treatment group for each frog to ensure that any differences in muscle performance were not due to whether the left or right leg was used. For the control group, gastrocnemius muscles were continuously bathed in oxygenated Ringer's solution at room temperature (21 ± 0.5 °C). The solution was oxygenated with a

bubbler and had an average dissolved oxygen reading of 8.0 ± 0.2 mg/L, which represents a normoxic environment for this species (Tattersall & Boutilier, 1997). For the treatment group, gastrocnemius muscles were continuously bathed in standard Ringer's solution at room temperature (21 ± 0.5 °C). This solution was not bubbled and had an average dissolved oxygen reading of 4.5 ± 0.2 mg/L, which represents hypoxic conditions (Tattersall & Boutilier, 1997).

A PowerLab data acquisition system (AdInstruments, Colorado Springs, CO) was used to control the stimulator rod and analyze the data collected through the force transducer. Contractile force was measured in Newtons, and the preload of each gastrocnemius muscle tested was set at 0.75 N, to maintain preload consistency across all muscles. The effects of voltage stimulus amplitude and duration of stimulation (fatigue) on contractile force were observed in relation to dissolved oxygen treatment. The effect of voltage stimulus amplitude on contractile force was investigated by using a macro program that stimulated the muscle once per second, in increments of 0.05 V, from 0.05 to 1 V, for 20 seconds. The voltage required to elicit the first detectable contractile force was recorded, as well as the strength of the first contraction. The final contractile force after stimulation with 1 V was also measured. We used a different macro program to then induce muscle fatigue. Fatigue was measured by first assessing the voltage that induced maximal contractile force, then continually stimulating the muscle at that voltage for 30 seconds. The measured force at the end of the 30 seconds was recorded, as well as the time required for the contractile force to reach zero, if it reached zero before the end of 30 seconds. The maximum contractile force reached across the 30-second interval and the time at which the muscle reached maximum contractile force were also recorded. Both the effect of voltage stimulus amplitude and fatigue variables were measured on the same day, at 11 AM, 3 PM, and 7 PM, to determine if the dependent variables were affected by time in vitro.

We used a repeated measures analysis of variance (RM ANOVA) to analyze the effects of dissolved oxygen treatment and time point on all dependent variables. All analyses were conducted with SPSS for Windows, v. 27 (IBM Corp.), and we considered results to be statistically significant if $p \leq 0.05$.

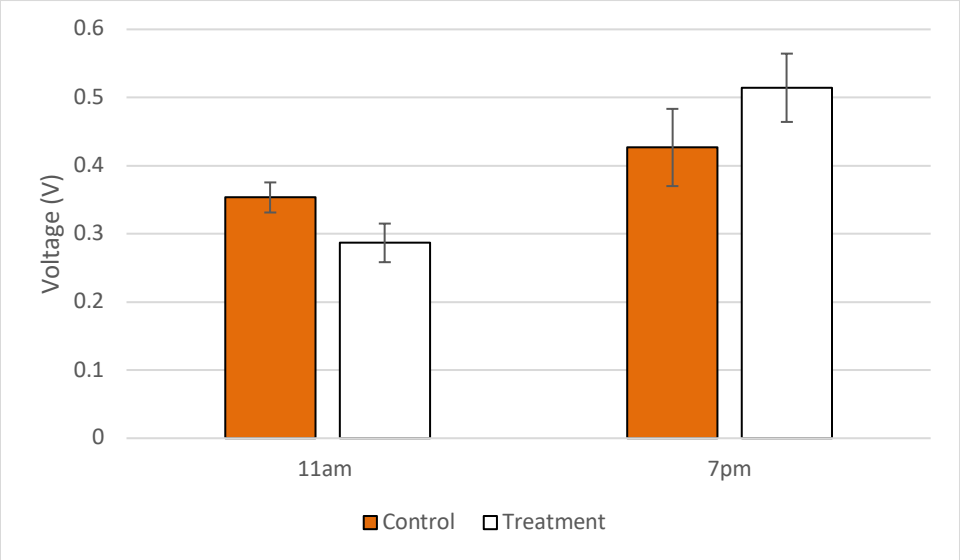
Results

Data for the 3 PM time point were transformed but could not conform to the assumption of homogeneity of variance ($p < .05$), so the data at this time point were not used in the final parametric analyses. Box's test of equality of covariance matrices indicated conformation to equality of covariances for most variables (all $p > .055$), but the force of first detectable contraction and the force at 1 V had to be log-transformed to conform to the assumption of homogeneity of variances (after transformation, p = .148 and .202, respectively).

Stimulus Voltage to Elicit First Detectable Contractile Force

We found that voltage needed to elicit the first detectable contractile force increased between 11AM and 7 PM ($F_{1,26} = 5.956, p = .022$) but there were no statistically significant effects of dissolved oxygen treatment ($F_{1,26} = 0.019$, $p = .184$) or the interaction between time point and dissolved oxygen treatment ($F_{1,26} = 0.756, p = .393$) (Figure 1).

Figure 1. Voltage Required to Elicit First Detectable Contractile Force



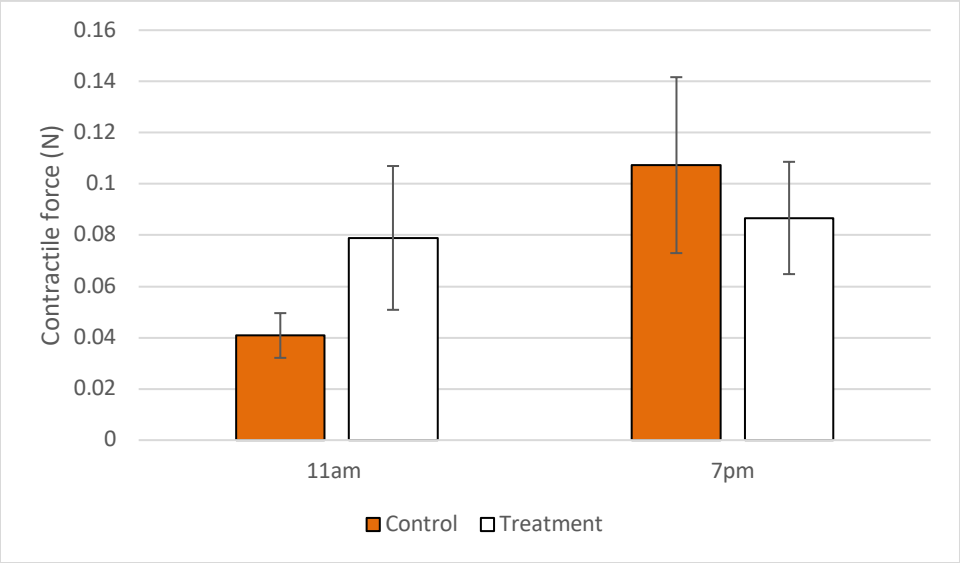
Note. No significant difference existed between the control (normoxic) and hypoxic oxygen treatments ($p = .184$) or in the interaction between dissolved oxygen treatment and time ($p = .393$); however, the voltage to elicit the first detectable contractile force differed between tests done at 11 AM and those done at 7 PM ($p = .022$).

Force of First Detectable Contractile Force

We found that the force of the first detectable contractile force decreased between 11 AM and 7 PM ($F_{1,26} = 7.532$, $p = .011$) but there were no statistically significant effects of dissolved oxygen treatment ($F_{1,26} = 0.192$, $p = .665$) or the

interaction between time point and dissolved oxygen treatment ($F_{1,26} = 0.263, p = .613$) (Figure 2).

Figure 2. Force of First Detectable Muscle Contraction

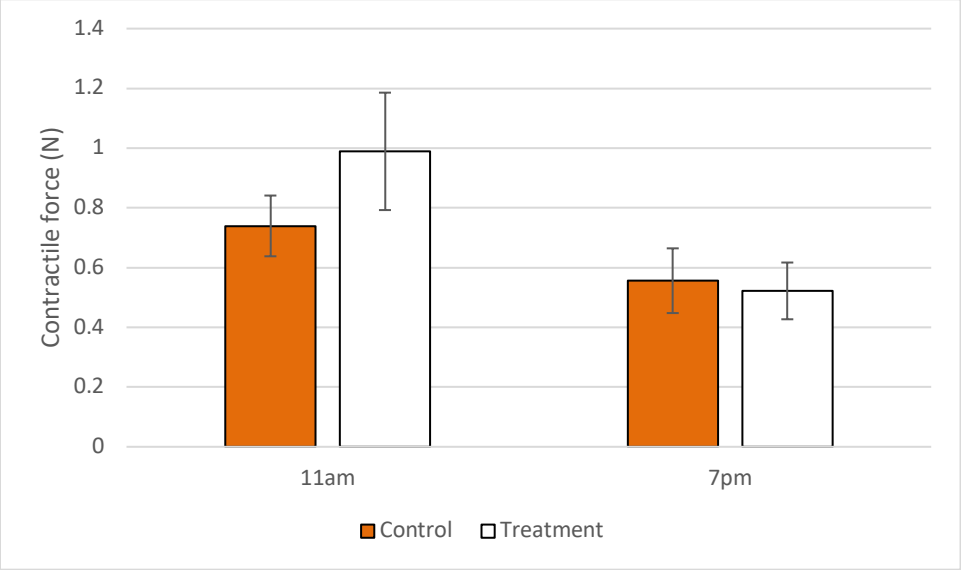


Note. No significant difference existed between the control (normoxic) and hypoxic oxygen treatments ($p = .665$) or in the interaction between dissolved oxygen treatment and time ($p = .613$); however, the force of the first detectable contraction differed between tests done at 11 AM and those done at 7PM ($p = .011$).

Force at 1 V Stimulation

We found that the force at 1 V stimulation decreased between 11 AM and 7 PM ($F_{1,27} = 4.634, p = .040$) but there were no statistically significant effects of dissolved oxygen treatment ($F_{1,27} = 0.273, p = .606$) or the interaction between time point and dissolved oxygen treatment ($F_{1,27} = 1.152, p = .293$) (Figure 3).

Figure 3. Contractile Force at Stimulation of 1 V

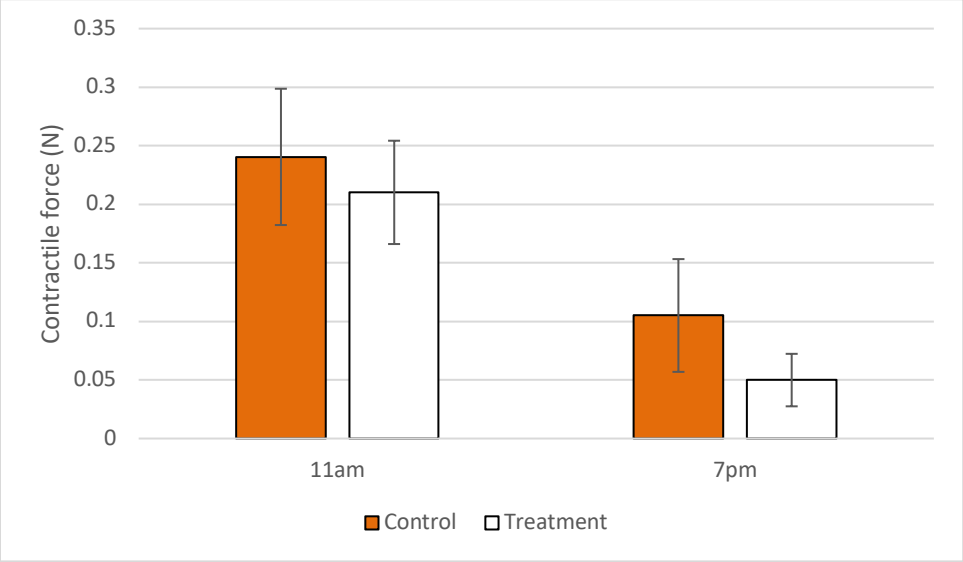


Note. No significant difference existed between the control (normoxic) and hypoxic oxygen treatments (p = .606) or in the interaction between dissolved oxygen treatment and time (p = .293); however, the force at stimulation of 1 V differed between tests done at 11 AM and those done at 7 PM (p = .040).

Muscle Fatigue: Contractile Force at the End of 30 Seconds

We found that contractile force at the end of 30 seconds of stimulation decreased between 11 AM and 7 PM ($F_{1,18} = 5.950, p = .025$) but there were no statistically significant effects of dissolved oxygen treatment ($F_{1,18} = 0.181$, $p = .676$) or the interaction between time point and dissolved oxygen treatment ($F_{1,18} = 0.120, p = .733$) (Figure 4).

Figure 4. Contractile Force at 30 Seconds of Stimulation (Fatigue)

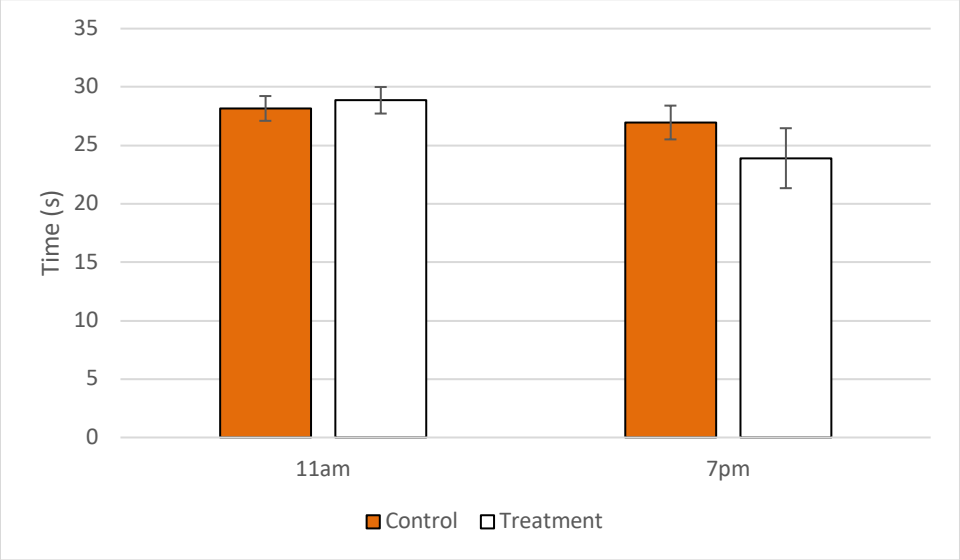


Note. No significant difference existed between the control (normoxic) and hypoxic oxygen treatments (p = .676) or in the interaction between dissolved oxygen treatment and time (p = .733); however, the time to reach force at 30 seconds of stimulation differed between tests done at 11 AM and 7 PM (p = .025).

Muscle Fatigue: Time Until Fatigue

We found that time until fatigue decreased between 11 AM and 7 PM ($F_{1,18}$ = 6.111, p = .024) but there were no statistically significant effects of dissolved oxygen treatment ($F_{1,18}$ = 0.062, p = .806) or the interaction between time point and dissolved oxygen treatment ($F_{1,18}$ = 0.760, p = .395) (Figure 5).

Figure 5. Time for Muscle to Reach Full Fatigue



Note. No significant difference existed between the control (normoxic) and hypoxic oxygen treatments (p = .806) or in the interaction between dissolved oxygen treatment and time (p = .396); however, the time to reach full fatigue differed between tests done at 11 AM and those done at 7 PM (p = .024).

Discussion

For contractile force with increasing stimulation, the voltage required to elicit the first detectable gastrocnemius contraction, strength of the first detectable contraction, and contractile force at 1 V did not show significant changes based on dissolved oxygen treatment. These values changed over time, however; after 8 hours, a more intense stimulus was required to elicit the first contractile force, and there was a decrease in the first detectable contractile force and force at 1 V stimulation. For variables related to fatigue, the amount of time required for gastrocnemius muscles to fatigue and the contractile force after 30 seconds of stimulation did not differ between the control and low dissolved-oxygen treatment groups; however, contractile force after 30 seconds of stimulation and time until fatigue did significantly decrease after 8 hours in vitro. While our hypothesis of decreased muscle performance due to a decrease in dissolved oxygen was not supported, there was a significant difference between tests done later in the day, which resulted in a decline of muscle performance after 8 hours in vitro.

Decreased strength of contraction and increased fatigue over time are consistent with previous studies showing that muscles in vitro are viable for

producing adequate contractile force for only a few hours (Smith & Meyer, 2020). Muscle viability loss is likely due to the lack of blood flow and vasculature that supply muscles with oxygen and nutrients necessary to carry out metabolic processes while also removing waste products (Smith & Meyer, 2020). Viability is not the only factor, as studies have shown that contractile force was also reduced significantly after 40 minutes of incubation of in vitro muscles (Croes & von Bartheld, 2007). Decreases in muscle viability and the strength of contractions over time have shown to be less severe in muscles incubated in oxygenated Ringer's solutions (Croes & von Bartheld, 2007), but further research concerning the viability of muscles over time for measuring contractile force and fatigue may be beneficial. It is likely that ischemic conditions are the cause of decreased muscle performance and viability over time and could possibly be prevented by performing this study in vivo with a continuous blood supply.

We observed no effect of the use of a hypoxic Ringer's solution in decreasing muscle viability, nor in decreasing the strength of muscle contractions or increasing fatigue. The ineffectiveness of the dissolved oxygen treatment on the variables measured in this study is consistent with some other studies that used similar techniques. For example, Croes and von Bartheld (2007) did not report significant effects of the use of oxygenated Krebs buffer on twitch force in lateral gastrocnemius and superior oblique muscles of juvenile white leghorn chickens (Croes and von Bartheld, 2007). In comparison, some species have exhibited an increase in muscle performance with the use of oxygenated systems. Atlantic cod (*Gadus morhua*), for example, exhibited a 41% decrease in swimming speed under deep hypoxic conditions (4.3 kPa of oxygen; Herbert & Steffensen, 2005), which could be related to a decrease in the strength of muscle contractions. Similarly, golden grey mullet (*Liza aurata*) demonstrated a 25% decrease in escape behavior in response to predatory stimuli under hypoxic conditions (10% air saturation; Lefrançois et al., 2005), which could be attributed to a decrease in muscle metabolic activity. In the previous two studies, the difference between normoxic and hypoxic was much greater than in our study, which may explain why they arrived at significant differences whereas we did not. It may be that we did not have sufficient resolution to distinguish between our two dissolved-oxygen treatment groups.

The inconsistency of our data with other studies could be attributed to other differences in experimental methodology. For example, in our study, muscles were wrapped in paper towels soaked with normoxic or hypoxic Ringer's solution and bathed during testing. In other studies, the experiment was performed while the muscles were submerged in the Ringer's solution (Croes & von Bartheld, 2007), which may have provided a more consistent dissolved-oxygen environment to the muscles. There were also differences in the methodology for oxygenating or deoxygenating the Ringer's solution; there has not been a universally accepted method for manipulating oxygen in this type of experiment. Croes and von Bartheld (2007) used a 95% O_2 and

5% CO_2 gas to oxygenate the buffer, whereas we used atmospheric gas, which is about 21% O_2. This compositional difference could have affected oxygen or other dissolved gas levels of Ringer's solutions, thus affecting muscle performance. Another technique to create a hypoxic environment is to bubble the Ringer's solution with nitrogen and seal the reservoir where the muscle is housed (Penghan et al., 2014). The method of deoxygenating rather than oxygenated solutions is another source of variation that may have contributed to the differences in the effect of hypoxia found in our and other studies.

It is possible that the metabolic rate of the frog is a major contributor to the extent of cutaneous respiration. The proportion of cutaneous oxygen uptake may positively correlate with metabolic rate. It is also plausible that frogs may rely on cutaneous respiration only when submerged for a longer time or when they are exposed to warmer temperatures that limit the dissolved oxygen content of water. Instead, the lungs may be the primary source of respiration when the amphibian is warm or breathing air. If any of the previous are important modulators among dissolved oxygen, cutaneous respiration, and muscle performance, simply exposing a disembodied muscle to dissolved oxygen at room temperature may not change the characteristics of the muscle.

Decreased dissolved oxygen in aquatic ecosystems due to climate change has been affecting wildlife distribution and behavior. Specifically, variation in dissolved oxygen alters the distribution of aquatic populations, leading to behavioral and physiological changes in some species and disrupted food webs on a larger scale (Breitburg, 2002). Additionally, nitrogen concentrations in some aquatic systems are fluctuating because of human addition of fixed nitrogen from agriculture (Joyce, 2000), which in turn affects oxygen levels. Climatic and anthropogenic environmental change are affecting more and more aquatic ecosystems, and insight on how dissolved oxygen levels affect aquatic species could be beneficial in understanding and mitigating these issues.

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