Behavioural Processes 219 (2024) 105047

Contents lists available at ScienceDirect

Behavioural Processes

journal homepage: www.elsevier.com/locate/behavproc

Acute hypoxia exposure rapidly triggers behavioral changes linked to cutaneous gas exchange in Lake Titicaca frogs

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ARTICLE INFO

Keywords:
Ventilation
Cutaneous respiration
Pushup behavior
Frogs

ABSTRACT

Ventilation is critical to animal life—it ensures that individuals move air/water across their respiratory surface, and thus it sustains gas exchange with the environment. Many species have evolved highly specialized (if not unusual) ventilatory mechanisms, including the use of behavior to facilitate different aspects of breathing. However, these behavioral traits are often only described anecdotally, and the ecological conditions that elicit them are typically unclear. We study one such “ventilation behavior” in Lake Titicaca frogs (*Telmatobius culeus*). These frogs inhabit high-altitude (i.e., low oxygen) lakes in the Andean Mountains of South America, and they have become textbook examples of cutaneous gas exchange, which is essentially breathing that occurs across the skin. Accordingly, this species has evolved large, baggy skin-folds that dangle from the body to increase the surface area for ventilation. We show that individuals exposed to acute hypoxic conditions that mirror what free-living individuals likely encounter quickly (within minutes) decrease their activity levels, and thus become very still. If oxygen levels continue to decline, the frogs soon begin to perform push-up behaviors that presumably break the low-oxygen boundary layer around skin-folds to increase the conductance of the water/skin gas exchange pathway. Altogether, we suspect that individuals rapidly adjust aspects of their behavior in response to seemingly sudden changes to the oxygen environment as a mechanism to fine tune cutaneous respiration.

1. Introduction

Ventilation describes the mechanical process by which individuals force air or water across their respiratory surface to sustain gas exchange (Boggs, 2002; Brainerd, 1999; Feder and Burggren, 1985; Milsom, 1989). Interestingly, many species have evolved unique behavioral traits to support regular ventilation. One of the best examples comes from sharks, tuna and other boney fishes that use ram ventilation to breathe by opening their mouth while swimming to force water across their gills (Roberts, 1975). For some species, the process of ram ventilation is essential to sustain gas exchange (Brown and Muir, 1970; Sepulveda et al., 2003), whereas other species use it facultatively to decrease costs associated with ventilating the gills (Roberts, 1975). The animal world is full of other examples of ventilatory behavior, such as the ability of some species to dive under water with air bubbles from which they can breathe (Boccia et al., 2021; Flynn and Bush, 2008; Seymour and Matthews, 2013) or the ability of other species to perform unusual body movements that help enhance the flow of water or air along a respiratory surface (Frakes et al., 2021; Genkai-Kato et al., 2000; Harlan and Wilkinson, 1981; Knight and Gaufin, 1963; Nagell, 1973). However, ventilatory behavior is most commonly described anecdotally, and thus much about it remains unclear. We know, for instance, little about either the natural environmental conditions that can trigger the production of ventilatory behavior or the way that such behavior is intertwined with other actions to help build a broader ventilatory strategy.

Here, we study behavioral strategies used to support ventilation in the Lake Titicaca frog (*Telmatobius culeus*). Like the ≈60 species within this genus (De la Riva et al., 2010), Lake Titicaca frogs live exclusively in high-altitude lakes that are scattered across the Andean Mountains in South America. Waters in these lakes are cold, ranging from 11 ℃ to 17℃ (Hutchison et al., 1976; Muñoz-Saravia et al., 2018), and have a relatively low oxygen content because of the reduced partial pressure of oxygen at high altitude and the high total dissolved salt concentration in the water (≈5–7 mg/L at 15℃) (Achá et al., 2018; Iltis et al., 1992;

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https://doi.org/10.1016/j.beproc.2024.105047
Received 7 December 2023; Received in revised form 7 March 2024; Accepted 14 May 2024

Vincent et al., 1985). In Lake Titicaca itself, limnological studies show that water oxygen can drop far below these levels at certain depths and during summer stratification, hitting values as low as 1–2 mg/L (Iltis et al., 1992; Lazzaro, 1981; Vincent et al., 1985). In these warmer periods, bottom layers that are isolated from water circulation even become anoxic (Iltis et al., 1992). One of the main ways that Lake Titicaca frogs have adapted to this environment is by evolving a system of near-obligate cutaneous respiration, meaning that individuals almost exclusively breathe through their skin (Hutchison et al., 1976). Indeed, these frogs rarely breach the lake's surface to breathe, doing so only when water oxygen levels are exceptionally low and/or when individuals are experiencing severe hypoxemia (Hutchison et al., 1976). It is therefore unsurprising that lungs of Lake Titicaca frogs are greatly reduced and contain underdeveloped alveoli (Hutchison et al., 1976). This species has instead highly vascularized skin that hangs in deep folds from the body and its appendages (Fig. 1A; Hutchison et al., 1976). Functionally, these folds increase the surface area of the skin, which is the primary site for gas exchange to occur. The original study that explores these respiratory adaptations in Lake Titicaca frogs also reports that some individuals exhibit interesting bobbing and/or pushup behavior when they are physically and forcibly restrained under extreme hypoxic water (Hutchison et al., 1976). It was hypothesized that such behavior might augment ventilation across the skin's surface, and therefore allow individuals to better withstand a low oxygen environment. However, this idea was not rigorously tested. It is difficult, for example, to distinguish these actions as elective forms of natural ventilation behavior, as opposed to stress-related behaviors that are elicited by physical restraint and near-suffocation (Knight and Gaufin, 1963). In this way, more ecologically relevant tests are needed to determine exactly how Lake Titicaca frogs might use basic motor acts to facilitate skin ventilation when water oxygen levels fluctuate as they might in a lake habitat.

We conduct such work by subjecting individuals from a population of Lake Titicaca frogs to an ecologically relevant respiratory challenge, in which they are acutely exposed to hypoxic conditions. As described above, dissolved oxygen gradients in Lake Titicaca vary greatly across the year, depending on lake depth and a host of other ecological factors (Achá et al., 2018; Vincent et al., 1985). While Lake Titicaca frogs are often found 1–3 m below the water surface in the shallows where dissolved oxygen levels are ≈6–7 mg/L (Achá et al., 2018; Iltis et al., 1992; Vincent et al., 1985), individuals can also be found much deeper in the lake (Muñoz-Saravia et al., 2018). Past studies, for instance, indicate

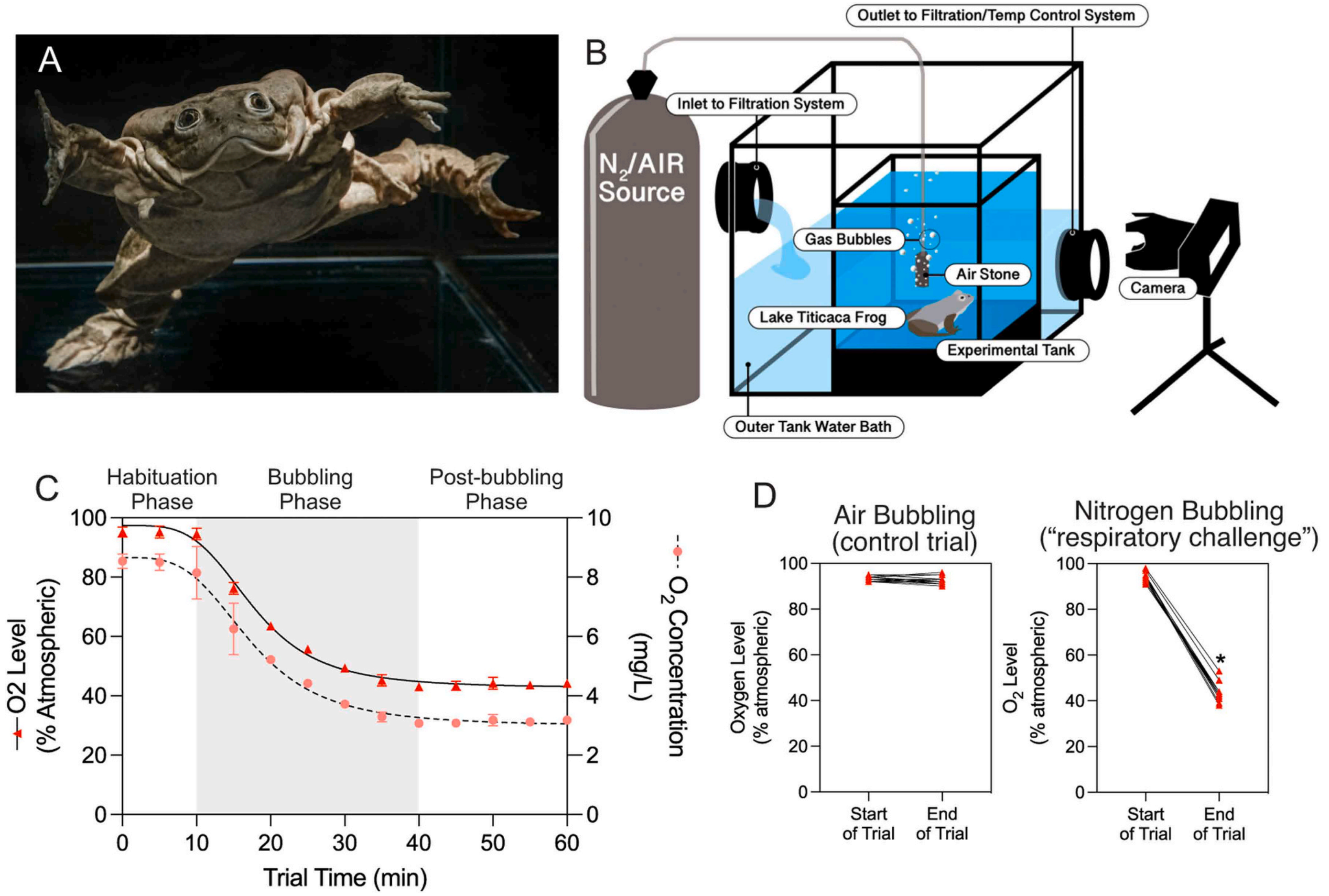

Fig. 1. (A) An adult Lake Titicaca frog (*Telmatobius culeus*). (B) Schematic of our experimental set-up, in which we subjected adult frogs to either a respiratory challenge by dropping water oxygen content in the experimental tank or a control trial (no change in water oxygen level). See methods for details. (C) Oxygen titration curve in response to bubbling nitrogen through the water of the observation tank during the respiratory challenge. There are two lines that represent the different ways we measure change in water oxygen—one line with red triangles that represents atmospheric oxygen (%, left vertical axis) and the other line with pink circles that represents oxygen concentration (mg/L, right vertical axis). The 60-min observation session was split into three phases, including the initial *habituation phase*, the secondary *bubbling phase*, and the final *post-bubbling phase*. Symbols represent average oxygen levels (per cent atmospheric or mg O_2/L) measured in 5-min increments across the entire duration of the trial, with error bars representing +/- 1 standard deviation. Note that some error bars appear absent, but instead are small and thus reflect little variation. (D) Oxygen levels in experimental tank at the start of the trail and at the end of the trial when air is bubbling through the water (control trial) or when nitrogen is bubbled through the water (respiratory challenge). Lines connect paired data points (water samples measured in the same trial), with the asterisk (*) representing a significant decrease in oxygen level ($p<0.001$).

that fisherman routinely find frogs at the bottom of the lake (Hutchison et al., 1976), where oxygen levels reach ≈1–2 mg/L (Iltis et al., 1992; Lazzaro, 1981; Vincent et al., 1985). We expect that frogs swim between these different areas with relative ease, meaning that they likely experience sudden shifts in dissolved oxygen levels. Indeed, there are several reasons why frogs might show different preferences for shallow and deep waters at different times of the day, month, or year (food availability, predator risks, etc.). In this way, we hypothesize that frogs evolve mechanisms to rapidly adjust their behavioral milieu to fine-tune the process of cutaneous ventilation through time and space. We therefore describe in our current study i) the behaviors that individuals produce in their aquatic environment and ii) which of these behaviors are modified in response to a sudden decline in dissolved oxygen. Importantly, this decrease in oxygen levels likely mirrors the drop individuals would experience if they swam from the shallows to the deep portions of the lake's water column. Further, we conduct this manipulation on a relatively rapid timescale (30 min), which is likely reasonably consistent with what a frog would normally experience if they traveled between aquatic environments with varying oxygen levels (i.e., swimming from the lake's surface to its depths). As such, we can determine how behavior is adjusted to likely augment cutaneous respiration.

2. Methods and materials

2.1. Animals

We studied a reproductively active population of endangered Lake Titicaca frogs (*Telmatobius culeus*; IUCN SSC Amphibian Specialist Group, 2020) that were kept in the Aquarium House at the Vienna Zoo (Schönbrunn), Austria (≈600 m above sea level) as part of an international *ex situ* conservation-figbreeding program. Frogs were maintained in large groups in four separate aquaria. Three of these aquaria were 126 L (60×70×30 cm), with each containing either eight (six male, two female), seven (all male), or four (two male, two female) individuals. One of these aquaria was 833 L (170×70×70 cm), and it contained 18 individuals (six males, twelve females). We maintained water temperature at ≈15 ̊C (range: 14.9–16.5 °C), using a singular water circulation system. Dissolved oxygen levels in these aquaria varied from 8.5 to 9.1 mg O2/L, which corresponds to roughly 94–97 % of the expected oxygen concentration in equilibrium with air at sea level. Frogs were fed worms (*Lumbricidae*) every morning between 08:00 and 11:00. For our study, we used a total of 23 individuals (n=12 males, n=11 females) that ranged in size from 29.9 g to 58.8 g (mean=40.4 g).

2.2. Ethical note

The ethical standards of our project adhere to principles outlined by the *Guidelines for the Ethical Treatment of Nonhuman Animals in Behavioural Research and Teaching* (Animal Behaviour, 2024). As described above, we study the behavior of frogs that are acutely exposed to hypoxic conditions. Importantly, the low levels of dissolved oxygen that these animals experience are always well within the range of dissolved oxygen levels that individuals would otherwise naturally encounter in the wild (Achá et al., 2018; Iltis et al., 1992; Vincent et al., 1985). In fact, past work indicates that certain parts of Lake Titicaca where frogs live (Hutchison et al., 1976) contain levels of dissolved oxygen that are much lower than what we expose frogs to in our current experiment (Iltis et al., 1992; Lazzaro, 1981; Vincent et al., 1985). Furthermore, we always ensured that individuals were free to breech the water's surface at any time during the respiratory challenge, so that they could breathe with their lungs. We also constantly monitored each frog during the entire trial so that we could remove it from the experimental set-up if it showed signs of undue or extreme stress (e.g., constant lung breathing, floating at the surface of the water, bloating, etc.); however, this was never the case (see results). Thus, no frog in our study was ever at risk of suffocation, even if individuals did experience mild stress when oxygen levels declined (though, such stress is presumably similar to that which frogs would experience if they encountered low-oxygen lake environments). With these points in mind, it is highly unlikely that frogs in our study experienced any risk of harm. Indeed, all animal care procedures followed guidelines set forth by the Federal Ministry of Health, Austria (BGBl. II Nr. 486/2004; BGBl. I Nr. 118/2004), and all research was approved by the appropriate institutional authorities at the Vienna Zoo.

2.3. Experimental design and set-up

To study how water oxygen levels influence the behavior of Lake Titicaca frogs, we subjected individuals to an acute respiratory challenge in which we experimentally quickly decreased dissolved oxygen levels in the frogs' immediate aquatic environment. This approach is widely used in studies of respiratory physiology in aquatic species, particularly in work that explores how individuals cope with incidents of hypoxia (Brill et al., 2015; Jiang and McGaw, 2023; Pelster et al., 2018; Porteus et al., 2014). In our study, we subjected control frogs to the exact same conditions, but we did not alter the oxygen content of the water. We ran both types of trials (respiratory challenge and controls) by placing a single frog in an observation aquarium (25 L, 40×25×25 cm) for a period of 60 minutes. We then partitioned this block of time into three distinct phases. We called the first phase the '*habituation phase*' which lasted 10 minutes and allowed individuals to adjust to the observation tank before oxygen levels were manipulated (or not, in the case of controls). We called the second phase the '*bubbling phase*;' it lasted 30 minutes, and during this time we bubbled either i) pure nitrogen gas or ii) atmospheric air (via an air pump) through an air stone (rate=0.75 liters/minute). Importantly, bubbling nitrogen through water decreased the dissolved oxygen content (see below for validation), whereas bubbling air through the water kept the dissolved oxygen content at atmospheric equilibrium. Finally, we called the third phase the '*post--bubbling phase*;' it lasted 20 minutes, and during this time we stopped bubbling gas through the water and simply observed the frog as it experienced hypoxic or normoxic conditions.

We ran frogs through these trials one at a time, and each individual was assigned at random to experience either the respiratory challenge or control trial. In total, we ran 33 separate trials, with 16 respiratory challenge trials and 17 control trials. Note that 11 frogs participated in the study only once, and thus they experienced only one of the two trial types. However, 11 other individuals participated in the study twice. These individuals always experienced both the respiratory challenge and the control trial, with the first condition getting assigned at random and the second trial occurring no sooner than 24 hours after the first.

Importantly, our experimental set-up (Fig. 1B) was designed to allow an observer to clearly video-record all behaviors of the focal frog during the 60-minute trial. At the same time, we were able to control water temperature and oxygen content independently of each other. Accordingly, we filled the observation aquarium with oxygenated water from the circulating water system of the temperature-controlled housing aquaria, and we then placed this water-filled observation aquarium within a larger tank that was connected to the same water system. This set-up prevented water exchange between the observation aquarium and larger tank, while also ensuring that the observation aquarium held the same temperature as the water in the larger tank (≈15°C).

Each frog was video recorded for the entire 60-minute trial, using a digital camera (SONY RX10II). Subsequently, we measured snout-vent-length (mm) and weight (g) and photographed individual distinct back patterns for identification before returning the frogs to their housing aquaria.

2.4. Validation of respiratory challenge

We verified that bubbling nitrogen gas through the water of the observation aquarium decreased dissolved oxygen levels. To do this, we ran experimental trials with a frog in the observation aquarium, measuring oxygen levels every 5 minutes using a Milwaukee MW600 PRO Dissolved Oxygen Meter. We replicated this 7 times, creating an oxygen "titration curve" that shows how levels of this gas change during our manipulation in the *Bubbling Phase* (Fig. 1C). This curve reveals that i) bubbling nitrogen into the water significantly reduces the oxygen levels in the water of the observation aquarium (oxygen levels measured by % atmospheric: $F_{1.69}$=6633, $p<0.001$; oxygen levels measured by mg/L: $F_{1.69}$=1939, $p<0.001$); ii) there is *very little* variation in dissolved oxygen level among trials during the experimental decrease, and iii) there are consistently low levels of dissolved oxygen during the *Post-bubbling Phase* of the trial (≈50% atmospheric levels or ≈2.6 mg/L O_2). Frogs subjected to our respiratory challenge therefore encountered nearly identical conditions across independent trials, in that oxygen levels decreased at the same rate and stayed at this same low level until the end of the trial.

We conducted a second validation, in which we did not keep a frog in the observation aquarium while we bubbled nitrogen through the water and measured its dissolved oxygen levels. The presence of a frog did not alter the oxygen concentration at the end of the post bubbling phase (Mann-Whitney U, U=3, p=0.232). We did not compute similar oxygen titration curves when air was bubbled through the water, instead of nitrogen; however, we did verify that the oxygen concentration of the water in the observation aquarium did not show a difference when measured at the beginning and end of these control trials (Fig. 1D; W=-51, p=0.90). As expected, we did find a statistically significant drop in oxygen levels in the experimental group when we conducted a similar analysis (Fig. 1D; W=-120, $p<0.0001$).

2.5. Behavioral quantification and statistical analysis

We characterized the behavior of each frog during all trials. To do this, we created five different behavioral categories that describe the activities that we saw. These include: i) *General Activity*, ii) *Pushup Behavior*, iii) *Lung Breathing Behavior*, iv) *Parachuting Behavior*, and v) *Paddling Behavior* (Table 1). Importantly, we created these categories to be mutually exclusive of each other; thus, pushups, lung breathing, parachuting, and paddling were not counted as forms of general activity. This means that our behavioral variables can be viewed as independent from each other. Behaviors were scored blindly with respect to whether individuals were subjected to the respiratory challenge. As such, we analyzed each recording in 2-minute intervals, in which we counted the total number of times a frog performed each of the behaviors described in Table 1. Importantly, we believe that the so-called "bobbing" behavior previously reported by Hutchison et al. (1976) is similar to pushup behavior we describe herein.

Table 1
Ethogram of main behaviors that frogs show during our experiment.

Behavior	Definition	Supplemental Video Number
General Activity	A frog performs a repeated series of coordinated strokes with its arms and/or legs while in place or moving about the aquarium.	1
Pushup	A frog sitting motionless on its four limbs, then uses its two forelimbs to suddenly push itself off the ground and propel its body ≈2 cm upwards in the water column. The frog then remains still as it floats back down to the substrate.	2
Lung Breathing	The frog lifts its mouth and/or nostrils above the water's surface and then re-submerges its head completely.	3
Parachuting	The frog swims to the top half of the tank, then remains motionless with its limbs limp and extended while slowly floating down to the substrate.	4
Paddling	A frog performs a single or short series of uncoordinated kicks with its legs and/or strokes with its arms while in place.	5

For frogs subjected to the respiratory challenge and the control treatment, we compared behavior among the experimental phases (habituation, bubbling, and post-bubbling). To do this analysis, we ran generalized linear mixed models (GLMMs) using the *lme4* package (Bates et al., 2014) in RStudio (Team, 2022) with both experimental treatment and phase as fixed factors. In each model, we included an interaction term between these two variables. We included frog ID (individual identification) as a random variable in all our models. We followed up significant main effects and/or significant interactions with Tukey post-hoc comparisons. To ensure that behavioral data were normally distributed, we log transformed [LOG(X+1)] all values before running our models.

In a final set of analyses, we fit various models to the behavioral data that were found to change across phases and in response to the respiratory challenge (see also results). When possible, we compared salient parameters of these models; however, if this was not possible because a model of one group failed to converge, then we compared the parameters of the other model to zero (0) values. These analyses were run in GraphPad Prism (v. 9.5.1).

3. Results

Lake Titicaca frogs changed many of their behaviors across trial phases, regardless of whether we decreased oxygen levels. The most pronounced effect occurred with respect to general activity (Fig. 2A; χ^2_2=212.7, $p<0.001$), which was greatest in the habituation phase compared to either the bubbling phase ($p<0.001$) or the post-bubbling phase ($p<0.001$). Activity levels in these latter two phases were statistically indistinguishable from each other (p=0.854). Additionally, we found significant effects of trial phase on pushup behavior (Fig. 2B; χ^2_2=51.32, $p<0.001$), lung breathing behavior (Fig. 2C; χ^2_2=13.01, p=0.001), and parachute behavior (Fig. 2D; χ^2_2=9.830, p=0.007). Despite these effects, however, post-hoc analyses did not uncover any significant contrasts between pairs of phases (all p values>0.05, see supplemental Table 1), likely because of phase-specific differences in behavioral variation among individuals. Only paddling behavior remained statistically indistinguishable among the trial phases (Fig. 2E; χ^2_2=2.850, p=0.241).

As expected, we found that lowering dissolved oxygen levels in the observation tank led to more frequent pushup behavior (Fig. 2B; χ^2_2=25.73, $p<0.001$), but did not impact other behaviors (Fig. 2A, general activity: χ^2_2=2.880, p=0.090; Fig. 2C, lung breathing: χ^2_2=1.027, p=0.311; Fig. 2D, parachuting behavior: χ^2_2=1.539, p=0.215; Fig. 2E, paddling behavior: χ^2_2=2.45, p=0.118). We did, however, uncover significant phase × treatment interactions for general activity (Fig. 2A; χ^2_2=15.86, $p<0.001$) and pushup behavior (Fig. 2B, χ^2_2=52.12, $p<0.001$), which indicates that frogs exhibited phase-specific responses to our respiratory challenge. We used post-hoc analyses to clarify these effects further, showing that frogs subjected to our respiratory challenge decreased activity levels and increased pushup behavior specifically in the post-bubbling phase (activity: $t_{51.8}$=3.43, p=0.014; pushups: $t_{91.5}$=-8.79, $p<0.001$). We saw no effect of the respiratory challenge on these behaviors during either the habituation phase (activity: $t_{51.8}$=0.30, p=0.99; pushups: $t_{91.5}$=0.00, p=1.00) or bubbling phase (activity: activity: $t_{51.8}$=0.68, p=0.98; pushups: $t_{91.5}$=-0.77, p=0.97). Equally important, we uncovered no significant phase × treatment interactions for lung breathing behavior (χ^2_2=2.240, p=0.326), paddling behavior (χ^2_2=2.391, p=0.303), and parachuting behavior (χ^2_2=0.550, p=0.760).

In a final analysis, we modeled how frogs change their activity levels and pushup behavior during the respiratory challenge (Fig. 3). We started with activity level (Fig. 3A) by fitting one phase decay models to frogs exposed to our respiratory challenge and controls. Compared to control frogs, individuals subjected to the respiratory challenge showed

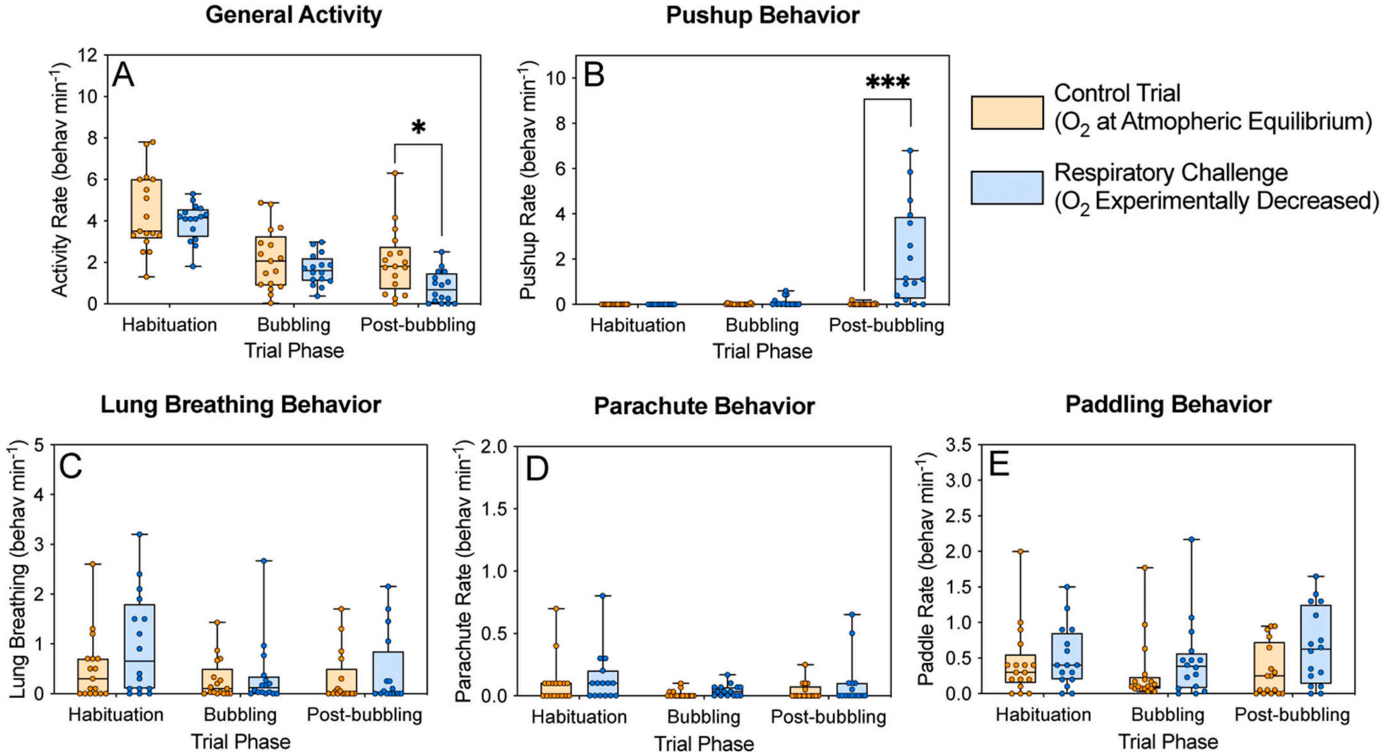

Fig. 2. Behavioral variables measured across the three different phases of either control trials (orange) or the respiratory challenge (blue). Dots represent behavior from a single individual, with boxplots and whiskers representing the distribution of the data in each phase and condition. The top and bottom of the box represents the upper and lower quartiles, respectively. The line in-between the top and bottom of the box represents the median, and the whiskers span the highest and lowest values accordingly. Significant differences between controls and respiratory challenge groups within a given phase are denoted by asterisks (*$p<0.05$, ***$p<0.001$).

significantly faster declines in activity (F_{1984}=4.60, p=0.032), as well as significantly lower activity plateaus (F_{1984}=41.99, $p<0.0001$). Next, we looked at pushup behavior by fitting four-parameter logistic regression models to our data (Fig. 3B). Notably, the model describing pushups in control frogs failed to converge, likely because individuals in this group rarely produced these movements. By contrast, our model for frogs subjected to the respiratory challenge was a good fit. We found, for example, a positive slope that significantly differed from zero (F_{1477}=250.60, $p<0.001$). We also found a ceiling effect of ≈6 pushups min^{-1} that was significantly different than 0 (F_{1477}=387.9, $p<0.001$).

In addition to summarizing these findings, Fig. 3 shows how changes to activity and pushup behavior temporally align with the concomitant decline in dissolved oxygen levels for frogs subjected to our respiratory challenge. Differences in activity levels begin to emerge as oxygen levels decline (Fig. 3A), whereas pushup behavior begins to appear once oxygen levels are ≈50% of atmospheric levels (≈5 mg O_2/L) (Fig. 3B). Amounts of pushup behavior increase relatively quickly, and they peak in accordance with the stabilization of low dissolved oxygen in the observation aquarium.

4. Discussion

Lake Titicaca frogs provide an iconic example of how animal respiratory systems can evolve. They breathe almost exclusively through their skin, and thus selection has increased the surface area through which gas exchange can occur by creating large, baggy skin-folds that dangle off the body and limbs (Hutchison et al., 1976). However, our current results add to this story by suggesting that "skin breathing" is not merely as passive as is often assumed. When frogs experience an acute drop in dissolved oxygen levels like they might if they occupy a deeper part of Lake Titicaca then individuals begin to modify their behavior in a manner that presumably influences cutaneous respiration (see below). The first sign of this effect can be seen through a decrease in activity, when oxygen levels in the water begin to drop. The second sign comes from results showing that individuals begin to perform pushups when oxygen levels are reduced to 50% of atmospheric levels, or ≈5 mg O_2/L. Pushup rates increase to ≈6 min^{-1} if low oxygen levels are maintained in the frog's environment. Importantly, because this respiratory challenge closely mirrors dissolved oxygen levels that these frogs would normally encounter in the wild when they move between locations within the lake or experience natural seasonal shifts in dissolved oxygen content, our findings likely reflect an adaptive behavioral strategy that individuals deploy to help augment cutaneous ventilation.

4.1. *Functional significance*

Why might Lake Titicaca frogs regulate body movement to mediate cutaneous respiration? With respect to the decrease in activity level, we suspect that this change in behavior is a strategy to conserve energy when gas exchange across the skin becomes more challenging due to the decrease in water oxygen levels (Booth and Feder, 1991). Lower levels of dissolved oxygen in the water would likely lead to a decrease in diffusion flux, particularly if a frog's internal dissolved oxygen levels do not deplete as quickly. This would mean that the frog's capacity for oxygen uptake is lowered (Feder and Burggren, 1985). In such a case, decreasing metabolic demand by suppressing activity might help offset any defect to cellular respiration that otherwise ensues (Biro and Stamps, 2010). Many diving mammals are known to use a similar strategy, as they increase gliding behavior and decrease limb movements during deep dives into the ocean (Davis et al., 2001; Williams et al., 2000).

When activity and movement are decreased, the flow of respiratory medium past the gas exchange organ remains critical. Ventilation can be maintained through pushups, a behavior that likely disrupts the formation of a low-oxygen boundary layer around the frogs' skin folds (Burggren and Feder, 1986) in a way that increases the conductance of the water/skin gas exchange pathways. Giant salamanders (e.g.,

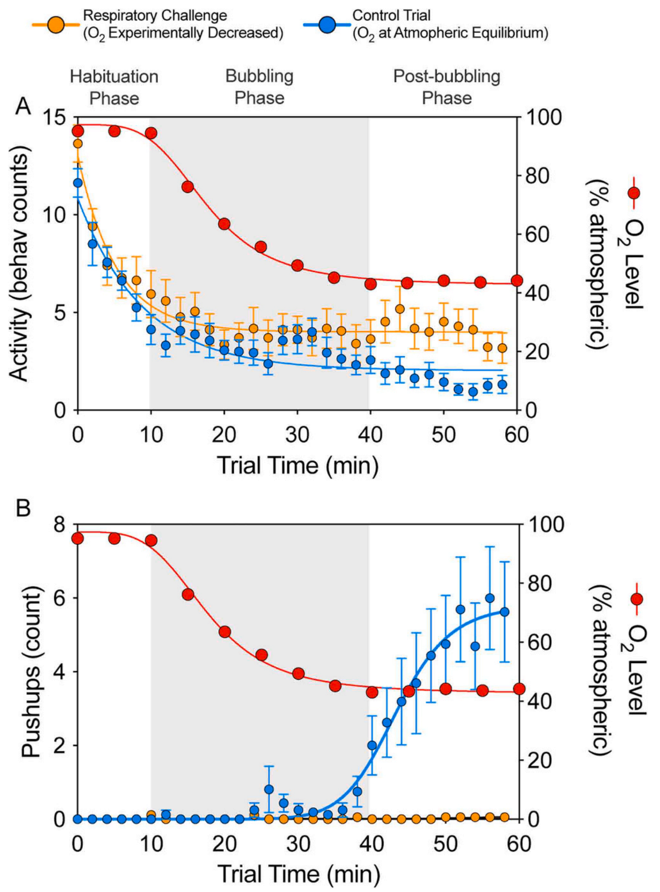

Fig. 3. Changes in (A) activity and (B) pushup behavior across both control trials (orange) and respiratory challenge trials (blue) presented in 2-min intervals. Each dot represents the mean count collected for the given condition (control vs. experimental) at the given time interval, with error bars representing +/- 1 SEM. Note that we overlay data showing the oxygen decline in the respiratory challenge (taken from Fig. 1C). Activity data (A) were fit with a one-phase decay model, whereas pushup data (B) were fit with a four-parameter logistic regression model.

Cryptobranchus alleganiensis) also have skin folds to increase surface area and are known to use a similar tactic by gently rocking when they are exposed to hypoxic waters (Harlan and Wilkinson, 1981; Ultsch, 2012). Past work suggests that this act flushes their small skin-folds with water, and thus boosts the partial pressure of oxygen in the blood by ≈5–7 mmHg (Boutilier and Toews, 1981a; Harlan and Wilkinson, 1981). While this increase may seem small, it is thought to be functionally significant since giant salamanders operate on the steeper portion of the animal's sigmoidal oxygen dissociation curve (Boutilier and Toews, 1981b). Indeed, this is where small differences in the partial pressure of oxygen confer relatively large differences in oxygen saturation of blood. We do not know the extent to which pushup behavior can increase oxygen blood levels in Lake Titicaca frogs; however, we expect pushups to have a similar, if not greater effect, than in giant salamanders. First, the skin-folds of the Lake Titicaca frogs are relatively larger than the folds of the salamander (Hutchison et al., 1976; Ultsch, 2012), implying that simple behavioral movements can have a greater impact on ventilation across the respiratory surface. Second, compared to giant salamanders, Lake Titicaca frogs have an especially low oxygen affinity of P_{50} (≈15 mm Hg) (Hutchison et al., 1976), which corresponds to the oxygen tension when hemoglobin is 50% saturated. In this way, Lake Titicaca frog blood has a relatively high affinity for oxygen, such that small increases in oxygen availability will have an outsized effect on oxygen binding in the blood.

Of course, we must also recognize that we did not perform refined physiological measurements of dissolved oxygen inside and outside the frogs before, during, and after pushup performance. This limitation leaves open alternate interpretations about the functional importance of pushup behavior. For example, lower levels of dissolved oxygen in the water would not, in theory, lead to a decrease in capacity for oxygen uptake through the skin if internal levels of dissolved oxygen fell at a similar rate. This is because the oxygen differential between a frog and its environment would remain the same, and by doing so have no appreciable effect on the dissolved oxygen gradient that supports the rate of gas diffusion. Future studies will therefore be needed to test gas exchange in the Lake Titicaca frog system.

4.2. *Movement as a behavioral strategy to support ventilation*

Our dataset also reveals that frogs appear to adjust activity levels and pushups without changing other forms of behavior. We found, for instance, that decreasing water oxygen levels had no appreciable impact on breathing behavior through the lung, which occurs when frogs breach the water's surface and inhale air. Lung breathing does sometimes occur in Lake Titicaca frogs, and it is thought to help individuals to facilitate gas exchange when dissolved oxygen levels get too low (Hutchison et al., 1976). In our study, frogs were free to breathe using their lungs, as we never restrained or trapped individuals underwater (as in Hutchison et al., 1976). This is an important point (even if it is a subtle one) because it means that frogs did not electively breathe through their lungs when oxygen levels dropped, but instead chose to suppress their activity levels and increase pushup behavior. We therefore hypothesize that our manipulation was not physiologically interpreted as "respiratory emergency," in which the risk of suffocation is high or imminent. Rather, we expect that the decrease in oxygen likely reflects a natural change in the environment that free-living frogs have evolved to manage. This idea lines up with research on these frogs in the wild, which shows that they occupy both shallow waters where oxygen levels are at or roughly below equilibrium with the atmosphere, as well deeper waters (Hutchison et al., 1976; Muñoz-Saravia et al., 2018) where dissolved oxygen levels get exceptionally low (Achá et al., 2018; Iltis et al., 1992; Lazzaro, 1981; Vincent et al., 1985). Additionally, we note that decreasing oxygen fails to trigger increases in paddling behavior, which is a basic form of locomotion (swimming). In other words, we did not see clear evidence that frogs became especially stressed in response to a respiratory challenge, or that they attempted to escape the situation by swimming away.

With these points in mind, it is also noteworthy that our modeling results highlight a degree of temporal precision with regards to changes in both activity and pushup behavior. Frogs, for example, first decrease their activity when dissolved oxygen levels drop, with noticeable changes in behavior occurring as oxygen content hits ≈80% of its atmospheric level. Frogs next increase pushup behavior, but they do so only after dissolved oxygen concentrations hit ≈50% of their atmospheric levels. Thus, we see signs of a clear ordered response as the environment changes. We hypothesize that this form of coordination reflects a more sophisticated and agile ventilatory strategy than previously ascribed to animals that might adopt movement to facilitate cutaneous respiration. The one notable exception is work done on stonefly nymphs (*Hesperoperla pacifica*, but formerly *Acroneuria pacifica*), which shows that individuals cycle through a range of different behaviors when water oxygen levels are chemically disrupted (Knight and Gaufin, 1963). Also interesting is that stillness (or low activity) is not one of these strategies.

If behavior is used to help fine-tune ventilation in Lake Titicaca frogs, then we imagine that individuals might construct other strategies based on the environmental conditions. For example, while we do not see instances of aerial (or pulmonary) respiration, we do know that these frogs will engage in such behavior (Hutchison et al., 1976). This may be something frogs do naturally when they are oxygen deprived, perhaps after spending a long time deep within the lake. When Hutchison et al.

(1976) reported attempted instances of aerial respiration it was in response to more prolonged and severe bouts of hypoxia. Although, in this study, frogs were often forcibly restrained or confined in water, which can be stressful and thus mask behaviors that individuals might otherwise show more naturally. Putting these considerations aside, our point is mainly that frogs likely have a plethora of flexible behavioral tactics that they can use to help breathe depending on the environmental context. This idea is broadly consistent with other studies that examine respiration in aquatic frogs, which show that individuals routinely modify how often they surface to breath in response to different environmental factors (Baird, 1983; Shannon and Kramer, 1988).

We suspect that behavior is used to influence gas exchange in wide range of species. This certainly includes other aquatic frogs, including the nearly 60 different species of *Telmatobius*; the behavior and ecology of these taxa largely remain a mystery,and only a few species have the characteristic baggy skin of the Lake Titicaca frog (Barrionuevo, 2017; De la Riva et al., 2010). Yet, the importance of behavior to gas exchange might even extend to taxa that are not obligate cutaneous respirators (Feder and Burggren, 1985). At the same time, it is also possible that behavior is used to mediate ventilation in species that do not engage in cutaneous respiration at all. This latter idea is provocative, but it is supported by work in humans and birds that suggests that limb positioning (Couser et al., 1992), body posture (Tickle et al., 2012), and/or behavioral performance (Mackelprang and Goller, 2013) can impact several aspects of breathing, including air flow mechanics throughout the respiratory system. How these animals might actively or intentionally use forms of behavior to enhance ventilation and reduce its metabolic demands is not yet clear, but the topic merits further investigation. Certainly, our current study illustrates that behavioral movement is likely a key part of ventilation in Lake Titicaca frogs, and such insight should be incorporated into the textbook story about this species and the evolution of its spectacular skin breathing abilities.

5. Conclusions

We find that “skin breathing” Lake Titicaca frogs use a complex mix of behavioral traits to manage respiratory challenges they encounter in their environment. In effect, frogs first decrease their activity levels, becoming stiller and more motionless as water oxygen levels decline. Then, if oxygen levels get too low, individuals perform pushups that likely ventilate the baggy skinfolds that droop from their body and facilitate cutaneous gas exchange. Frogs in our study did not increase lung breathing, nor did they show any overt signs of extreme stress that might manifest if individuals were becoming hypoxemic. Thus, we hypothesize that individuals adjust elective movements and produce stereotyped behavioral maneuvers specifically to augment ventilation through their spectacular skin-folds.

CRediT authorship contribution statement

Matthew J. Fuxjager: Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Doris Preininger:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Roland Halbauer:** Resources. **Nigel K. Anderson:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Jordan De Padova:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgments

We thank Elizabeth Brainerd for training on the methods used in this study, as well as comments on the final version of the manuscript. We also thank Elska Kaczmarek help assisting with the experimental setup, as well as Isaiah Dawkins for help conducting these experiments at the Zoo. Finally, we thank curator Anton Weissenbacher and the zookeeper team at the Vienna Zoo Aquarium House for project support. This research was supported by the National Science Foundation grant OSIE-1952542 (to MJF).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.beproc.2024.105047.

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