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Buoyancy control and air breathing in royal knifefish (*Chitala blanci*) and a new hypothesis for the early evolution of vertebrate air-breathing behaviors

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Abstract

We present the first description of inspiration-first air breaths in royal knifefish, *Chitala blanci*, a ray-finned fish known to use four-stroke air breaths. Four-stroke breaths are used by nearly all ray-finned fish species that use their gas bladder to breathe air and are the ancestral breath type of ray-finned fishes. Interestingly, one such species, *Amia calva*, is known to perform two distinct breath types. *Amia* use four-stroke breaths when they need more oxygen and performs inspiration-first breaths to restore buoyancy. We observed that *C. blanci* also performs inspiration-first breaths and tested whether the two breath types are performed for the same functions in *C. blanci* as they are in *Amia*. We recorded the frequency of each breath type when exposed to aquatic hypoxia and two conditions of oxygen availability. We found that *C. blanci* performed more four-stroke breaths (81% ± 15% of total breaths) than inspiration-first breaths when exposed to aerial normoxia but performed more inspiration-first breaths (72% ± 40%) than four-stroke breaths when exposed to aerial hyperoxia. These patterns match those described for *Amia* and indicate that *C. blanci* performs four-stroke breaths in response to oxygen depletion and performs inspiration-first breaths to maintain buoyancy. Few studies have examined the role of air-breathing in buoyancy regulation. Decreasing buoyancy, rather than oxygen availability, to stimulate air breaths may reveal that inspiration-first breaths are more common among fishes than we are aware. We consider this possibility and present a new hypothesis for the origin and early evolution of air breathing in vertebrates.

KEYWORDS

air-breathing, buoyancy, *Chitala*, evolution, fish

1 | INTRODUCTION

While, to a layperson, breathing air is seemingly antonymous to the idea of a “fish,” air-breathing is important to the survival and natural history of numerous fish species. The first air-breathing vertebrates were bony fish that evolved lungs as an air-breathing organ (ABO) approximately 430 million years ago, and these air-breathing fish are the ancestors of all terrestrial vertebrates and bony fishes alive today (Betancur-R et al., 2017; Brainerd & Ferry-Graham, 2005; Cass et al., 2013; Cupello et al., 2022; Liem, 1988; Longo et al., 2013; but see Damsgaard et al., 2020; Perry, 2007; Perry et al., 2001). There are approximately 50 extant fish species that breathe air using lungs or a respiratory gas bladder (Graham, 1997) and more than 600 that breathe air using a diverse array of other ABOs or respiratory epithelia (Damsgaard et al., 2020).

All air-breathing fishes are 'bimodal breathers' that possess gills, and their reliance on air-breathing to meet their oxygen demands varies from facultative to obligatory (Graham, 1997). Air-breathing species often have reduced gills, which is presumed to curb the loss of oxygen via the gills when in hypoxic water, but also reduces the utility of the gills for gas exchange and promotes reliance on the ABO (Graham, 1997).

The ability to extract oxygen from air is central to the concept of an air-breathing fish. Graham (1997) defined air-breathing fish as those that utilize gas exchange with the aerial environment for the purpose of respiration and to serve an adaptive function in the life history of the species. All fish have specialized receptors that are sensitive to the partial pressure of oxygen or carbon dioxide in the blood or in the environment (reviewed in Milsom, 2012). Within air-breathing fishes, these chemoreceptors stimulate air-breathing behavior in response to increased metabolic oxygen demand (resulting from increased physical activity or water temperature), aquatic hypoxia, and/or aerial hypoxia (reviewed in Florindo et al., 2018).

Although air-breathing fishes are defined by their ability to extract oxygen from the aerial environment, gas exchange is not the only function of air-breathing. Air-filled lungs and gas bladders (as well as other ABOs that store air) provide positive buoyancy, and air breaths can regulate buoyancy. Just as air-breathing frequency can be modulated to respond to changes in metabolic oxygen demand or oxygen availability, air-breathing activity can also be sensitive to ABO volume, that is, buoyancy (reviewed in Hedrick & Katz, 2016). Lungfish (*Protopterus aethiopicus* and *Lepidosiren paradoxa*), bowfin (*Amia calva*), and gar (*Lepisosteus oculatus*) have stretch-sensitive mechanoreceptors with firing rates that are sensitive to ABO volume (Delaney et al., 1983; Milsom & Jones, 1985; Smatresk & Azizi, 1987; reviewed in Hedrick & Katz, 2016). In *P. annectens*, *A. calva*, and *L. oculatus*, air-breathing responses are stimulated by ABO deflation and inhibited by ABO inflation (Johansen et al., 1970; Smatresk & Cameron, 1982; Pack et al., 1992; Hedrick & Jones, 1999; reviewed in Hedrick & Katz, 2016). In *Amia*, when researchers removed gas from the gas bladder, the resulting air-breathing frequency was significantly correlated with the volume of gas removed (Hedrick & Jones, 1999). There is also evidence that even when oxygen is abundant, *Piabucina festae*, *Hoplerythrinus unitaeniatus*, *Erythrinus erythrinus*, *Bronchis splendens*, *Corydoras aeneus*, and juvenile *Megalops atlanticus* breathe air to maintain buoyancy (Gee & Graham, 1978; Geiger et al., 2000; Graham et al., 1977; Kramer & McClure, 1980; Stevens & Holeton, 1978). Thus, there is strong evidence demonstrating that these fish modulate air-breathing frequency in response to changes in ABO volume.

Research in this area focuses on air-breathing frequency, but this focus neglects the other ways that air-breathing behavior can be adjusted to regulate buoyancy. Air-breathing fish might adjust the volume of gas expired and/or inspired during each breath or may use different types of breaths to increase or decrease buoyancy, where different breath types are distinguished by distinct sequences of airflow and cranial kinematics. It is also possible that some air-breathing fish modify both the volume of gas ventilated and the type of air breath used, or that some don't modify their air-breathing behavior at all (as may be the case for benthic species).

These possibilities have only been examined in one species, *Amia*, and these studies revealed that *Amia* use different breath types (Hedrick et al., 1994; Hedrick & Jones, 1993, 1999; Hedrick & Katz, 2016). To put the different air breath types in *Amia* in context, we will start with a description of the "four-stroke" breathing pattern.

The four-stroke breath is the most commonly observed breath type among actinopterygian fishes and has received the most research attention. Early diverging air-breathing actinopterygian fishes (including most species that use their lungs or gas bladder as an ABO) use a four-stroke breath, and so it is hypothesized to be the ancestral breath type of Actinopterygii (Brainerd, 1994a). The "strokes" of the four-stroke breath refer to either expansions or compressions of the buccopharyngeal cavity, by analogy with two-stroke and four-stroke piston engines. During a four-stroke breath, the first two strokes produce expiration: (1) gas is transferred from the gas bladder to the mouth, and (2) cleared from the mouth. The third and fourth strokes produce inspiration: (3) fresh air is taken into the mouth at the surface, and (4) pumped into the gas bladder via buccal compression (Figure 1).

Within *Amia*, Hedrick and Jones (1993; 1999) observed simpler breaths involving only inspiration or expiration (Figure 1c, d). In this paper, we refer to these as 'inspiration-first' breaths and 'expiration-only' breaths, to contrast them with the combined expiration and inspiration that occurs during a four-stroke breath.[1]

When exposed to aquatic hypoxia, *Amia* predominately use four-stroke breaths (Hedrick & Jones, 1993). In contrast, when the gas bladder is hyperinflated, *Amia* use expiration-only breaths, and when the gas bladder is deflated, *Amia* respond by almost exclusively using inspiration-first breaths (Hedrick & Jones, 1999). When exposed to aerial hyperoxia, *Amia* again took nearly exclusively inspiration-first breaths (Hedrick & Jones, 1993). Hedrick and Jones (1993) were unable to measure inspiratory volume, but they observed

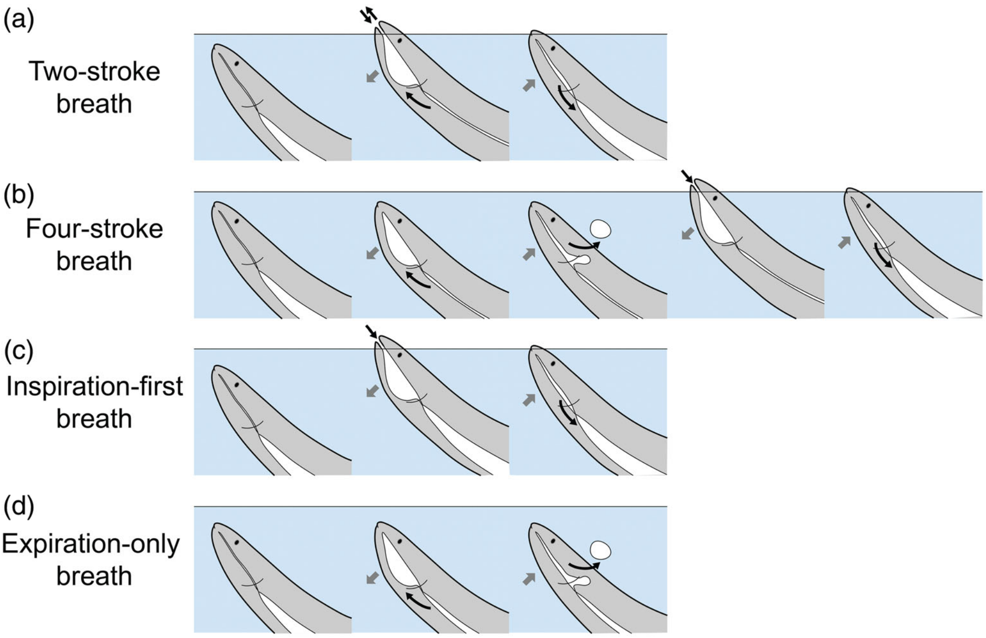


FIGURE 1 Types of air breaths. The sequence of buccal cavity expansion and compression and air movement is illustrated for (a) two-stroke, (b) four-stroke, (c) inspiration-first, and (d) expiration-first breaths. The fish-like animal depicted is intended to be generic, rather than a specific species. The two-stroke breathing pattern is present in lungfishes and amphibians that breathe with a buccal pump, and the four-stroke breathing pattern is present in ray-finned fishes. There is no known single species that takes both two- and four-stoke breaths.

that expiratory volume of the four-stroke breaths was not significantly different across the conditions tested. Thus, to regulate buoyancy, *Amia* uses varying breath types, including inspiration-first and expiration-only breaths, and they do not appear to modify the size of the breath.

It is unknown whether other air-breathing fishes regulate buoyancy in a similar way to *Amia*. The combined use of four-stroke, inspiration-first, and expiration-only breaths has not been described in any other air-breathing fishes. This would seem to indicate that *Amia* are unique in this regard. However, the rarity of observed inspiration-first and expiration-only breaths may, in fact, be an outcome of the experimental conditions used for studying air-breathing, rather than of the behavioral abilities of the species studied. Air-breathing fish are usually studied under conditions of aquatic hypoxia and aerial normoxia, but, under these conditions, *Amia* primarily used four-stroke breaths (Hedrick & Jones, 1993). If other air-breathing fish are indeed like *Amia*, this could explain why four-stroke breaths, not inspiration-first breaths, have been observed. In fact, before Hedrick and Jones (1993) discovered the use of inspiration-first breaths, the air-breathing behaviors of *Amia* had been studied in detail and yet had only described four-stroke breaths (Deyst & Liem, 1985; Liem, 1988; Liem, 1989; Randall et al., 1981). It is therefore possible, and perhaps probable, that other air-breathing species use four-stroke, inspiration-first, and expiration-only breaths, depending on whether the breaths are stimulated by oxygen-related or buoyancy-related functional demands.

If inspiration-first and expiration-only breaths are used by both actinopterygian and sarcopterygian species, they would be the only breath types known to be shared by both lineages. As noted above, four-stroke breaths are hypothesized to be ancestral to Actinopterygii, and similarly, "two-stroke breaths" (which also involve both expiration and inspiration of air, Figure 1) are hypothesized to be ancestral to Sarcopterygii (Brainerd, 1994a). Because there is strong evidence for a single origin of lungs (Cass et al., 2013; Funk, Breen, et al., 2020; Funk, Lencer, & McCune, 2020; Longo et al., 2013), and by extension, of air-breathing behavior in Osteichthyes, the split of four-stroke and two-stroke breath types across its two descendent phylogenetic branches has created seemingly intractable questions: What breath type is ancestral to Osteichthyes? Did four-stroke and two-stroke breaths evolve independently? Is one derived from the other? Or are they both derived from a third unknown breath type present in the ancestor of Osteichthyes? Inspiration-first and expiration-only breaths, and the possibility of their shared use across Actinopterygii and Sarcopterygii, present a new source of information for addressing these questions about the origin and early evolution of air-breathing in vertebrates.

We studied the air-breathing behaviors of royal knifefish (*Chitala blanci*), a facultative air-breathing species (Graham, 1997). Although the skeletal kinematics and muscle activity of the four-stroke breaths used by the clown knifefish (*Chitala chitala*) have already been studied in detail (Liem, 1989), we present the first description of inspiration-first breaths used by *Chitala*. We sought to determine whether four-stroke and inspiration-first breaths are stimulated by the same functional drivers in *C. blanci* as they are in *Amia*. We hypothesized that in *C. blanci*, like in *Amia*, four-stroke breaths are stimulated by a need for oxygen and that inspiration-first breaths are stimulated by a need for increased buoyancy. To test these hypotheses, we exposed *C. blanci* to two conditions (aquatic hypoxia and aerial normoxia; and aquatic hypoxia and aerial hyperoxia) and recorded the number of four-stroke and inspiration-first breaths that were used in each condition. We predicted that *C. blanci* would respond similarly to *Amia*, and as expected, *C. blanci* predominately used four-stroke breaths when exposed to aerial normoxia and predominately used inspiration-first breaths when exposed to aerial hyperoxia. We discuss the implications of these results for our understanding of the origin, early evolution, and functional significance of air-breathing.

2 | METHODS

Six royal knifefish (*C. blanci*) were acquired from the aquarium industry (Ocean State Aquatics, Coventry, RI, USA; and Aqua Imports, Boulder, CO, USA): Cb04 (standard length 433 mm), Cb05 (length not measured), Cb06 (325 mm), Cb07 (313 mm), Cb08 (250 mm), and Cb09 (253 mm). *C. blanci* were maintained on a diet of goldfish (*Carassius auratus*) and kept at 25–28°C. All husbandry and experimental procedures were approved by the Brown University Institutional Animal Care and Use Committee.

2.1 | X-ray video of air-breathing behavior

X-ray videos were taken of the air-breathing behaviors of two fish (Cb04 and Cb05; Figures 2 and 3). The fish were kept in custom-built acrylic aquaria with a narrow extension (75–100 mm wide, 300–400 mm long) that was designed to minimize the amount of water that the X-ray beams must travel through. Nitrogen gas was bubbled into the water to lower the aquatic oxygen concentration and stimulate the fish to breathe air. Lateral and dorsoventral X-ray videos (recorded at 200 or 500 frames s^{-1}) were captured using a custom biplanar X-ray system (Imaging Systems and Services, Painesville, OH, USA) and Phantom v10 high-speed cameras (Vision Research, Wayne, NJ, USA) at 100 mA and 68–115 kV. A standard grid was used to remove distortion introduced by the X-ray machines.

2.2 | Standard video of air-breathing behavior under experimental conditions

Four fish (Cb06, Cb07, Cb08, Cb09) were used to study the proportion of four-stroke and inspiration-first breaths used under two conditions of oxygen availability.

2.3 | Experimental setup

The fish were filmed in glass aquaria (600 mm × 310 mm × 420 mm), with approximately 230 mm water depth during video recording. The water surface was covered with a plastic grid (15 mm square openings) with a semi-circular opening (115 mm radius) through which the fish could breathe air. The opening was covered with half of a plastic dome that had a port through which a constant flow of gas was maintained during video recording. A curtain was hung during video recording so that the fish could not see movement in the rest of the room. In addition, a thin board was placed over the aquaria to make the tank more sheltered. No additional lighting was used, besides the ceiling lights in the room.

2.4 | Aquatic and aerial gas conditions

The fish were exposed to two experimental conditions: (1) aquatic hypoxia and aerial normoxia, and (2) aquatic hypoxia and aerial hyperoxia. Aquatic hypoxia is necessary to stimulate air breathing in *C. blanci* (a facultative air-breathing species).

The combination of aquatic hypoxia and aerial hyperoxia is intended to stimulate the use of air breaths but decouple oxygen availability from buoyancy. When Hedrick and Jones (1993) exposed *Amia* to aerial hyperoxia, *Amia* took nearly exclusively inspiration-first breaths. Hedrick and Jones hypothesized that exposing *Amia* to aerial hyperoxia caused the internal oxygen supply to remain high (therefore not stimulating four-stroke breaths), while oxygen diffused from the gas bladder, depleting its volume and stimulating inspiration-first breaths. (In fish, gas bladder volume becomes depleted because carbon dioxide is readily lost at the gills, rather than diffusing into the ABO, as occurs in terrestrial vertebrates.) Modeling of the physiology of *Amia* during air breathing provided strong support for this hypothesis (Hedrick & Katz, 2016). We sought to replicate these conditions and use exposure to aerial hyperoxia to inhibit the drive to obtain oxygen and isolate the drive to restore gas bladder volume.

Aquatic hypoxia was created by bubbling nitrogen into the tank. The bubbler was positioned in the corner of the tank so that nitrogen bubbles escaped through the plastic grate covering the water surface and did not bubble into the breathing dome. Nitrogen was bubbled in faster during experimental preparation to lower the aquatic oxygen concentration, and then the flow rate was slowed during video recording, maintaining hypoxia. The aquatic oxygen concentration was measured using a dissolved oxygen meter (HI 9142, Hanna Instruments, Woonsocket, RI, USA) before and after the start of video recording. The dissolved

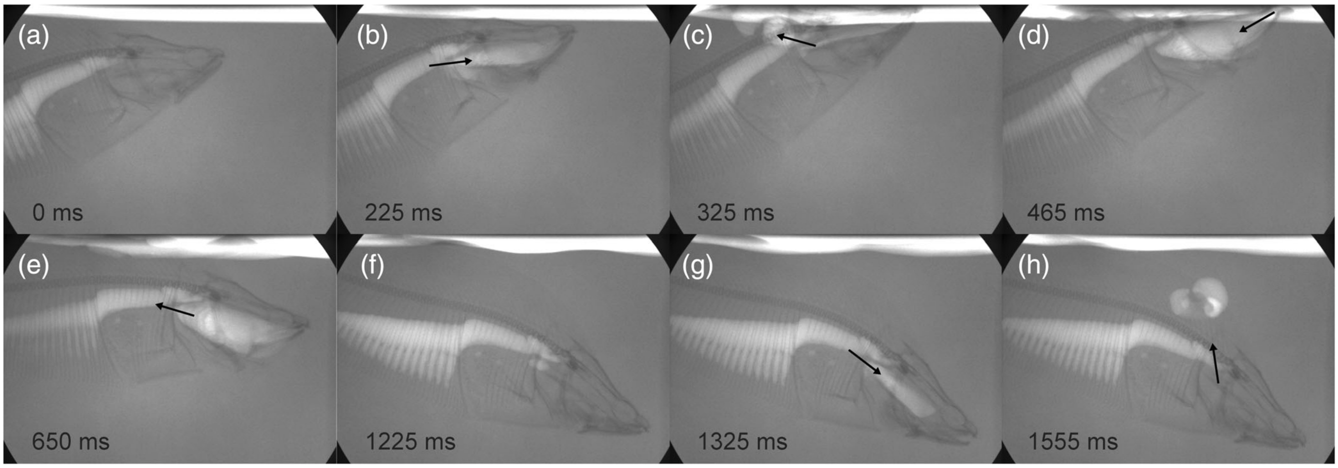


FIGURE 2 Frames from an X-ray video of *C. blanci* taking a four-stroke breath. During a four-stroke breath, the fish (a) approaches the surface, (b) expires gas from the gas bladder into its mouth, (c) compresses its mouth and expels expired gas as it rises above the surface, (d) expands its mouth to take in air, (e) descends below the surface, (f) compresses its mouth and inspires air into the gas bladder, (g) expands its mouth as excess gas is expired from the gas bladder, and (h) compresses its mouth to expel gas out of the opercula. Arrows indicate the direction of airflow.

oxygen meter was calibrated once with a zero-oxygen solution and then calibrated before experiments with a slope calibration in saturated air (100% DO). To maintain aerial normoxia, air was pumped into the breathing dome at a constant flow rate (approximately 300 mL/min). To create aerial hyperoxia, 100% oxygen was pumped into the breathing dome at a constant rate (approximately 600 mL/min during preparation and 300 mL/min during data recording).

The fish were also video recorded while exposed to aquatic and aerial normoxia to confirm their classification as facultative air-breathing fish.

2.5 | Standard video recording

On days prior to recording videos for data collection, the fish were given time to acclimate to the breathing dome. To do this, the experimental apparatus was set up and nitrogen gas was bubbled into the tank, allowing the fish to become comfortable taking air breaths in the breathing dome. The fish were observed to ensure that they were acclimating well to the experimental setup.

For data collection, the fish were video recorded for continuous 6-h sessions in each experimental condition. Each fish was exposed to different experimental conditions on different days. The recordings were made between 10:30 am and 8 pm. Videos were recorded at 10 frames s^{-1} using a Flare CX video camera (IO Industries, Ontario, Canada).

Four-stroke breaths were distinguished from inspiration-first breaths by visual indicators of expiration, which occur prior to inspiration in four-stroke breaths but do not occur in inspiration-first breaths. These visual indicators included (1) the collapse of the body wall adjacent to the gas bladder (Figure 3g, h), (2) depression and elevation of the hyoid as gas was transferred from the gas bladder into the mouth and then ejected via the opercula, and (3) release of gas bubbles out of the opercula. The fish tended to tilt slightly toward the front of the aquarium as they approached the surface and then bent to that side as they swam away from the surface after the air breath. Because of this, the expired gas often bubbled out of the opercular opening on the side of the fish not facing the camera. In these cases, the bubble could either be seen directly as it rose above the fish's head, could be seen indirectly via its reflection on the water's surface, or could be seen indirectly via the ripples that it created in the water's surface.

X-ray and standard video data were stored with their essential metadata in accordance with best practices for video data management in organismal biology (Brainerd et al., 2017). Examples of the video data are available in the public data collection "Air breathing videos for Kaczmarek and Brainerd, 2024" on the XMAPortal: https://xmaportal.org/webportal/larequest.php?request=CollectionView&StudyID=79&instit=BROWN&collectionID=26.

2.6 | Statistical analysis

We calculated the proportion of four-stroke breaths and inspiration-first breaths that were used in each experimental condition (i.e., aerial normoxia/aquatic hypoxia; and aerial hyperoxia/aquatic hypoxia condition). In addition, we fitted a binomial logistic mixed effect model to test whether the probability of the use of inspiration-first breaths was the same in both experimental conditions. The model included individual as random effect. All statistical analyses were performed using R v.4.3.1 (https://www.R-project.org/).

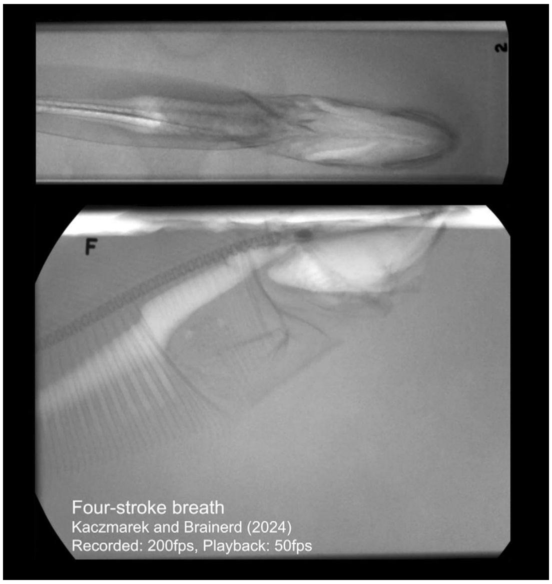
3 | RESULTS

3.1 | X-ray video of air-breathing behavior

We captured 16 inspiration-first breaths and 27 four-stroke breaths performed by Cb04, and 4 inspiration-first breaths and 3 four-stroke breaths performed by Cb05. We present these numbers to illustrate that we observed multiple inspiration-first breaths used by each individual. More breaths were recorded from Cb04 because we spent more time collecting data from this individual. The number of observations of each breath type used by each fish does not reflect the true proportion of breath types performed during X-ray video collection, as we did not capture every air breath performed, nor did we save video of every breath observed. The fish always ascended to the water surface, took a single air breath, and quickly descended. The fish ascended and descended with an upright body posture and did not descend with a C-start-like motion, as was seen in the standard videos.

3.2 | Four-stroke air breaths

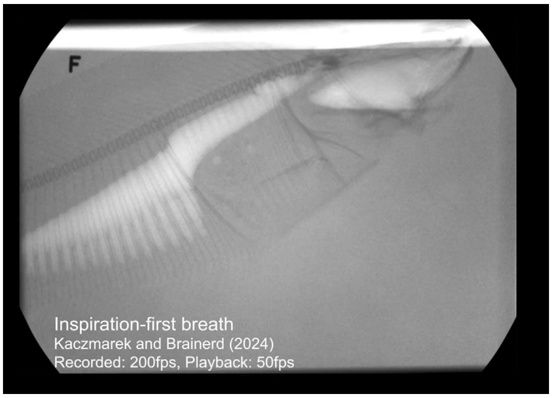
The main phases of the four-stroke breath are shown in Figure 2 (see Video 1). First, the fish approached the surface with its buccal cavity compressed. Before reaching the surface, the fish began transferring gas from the gas bladder to the buccal cavity, while the hyoid depressed. At a similar time that the buccal cavity reached peak expansion, the opercula abducted and gas rose out of the opercular openings. As the neurocranium rose above the water surface, the buccal cavity began compressing and gas continued to flow out of the opercula. The path of expired gas (out of the opercula) is similar to *Lepisosteus* (Rahn et al., 1971) and is unlike *Amia*, which expire gas via the mouth (Liem, 1989). The gas bladder continued to deflate while gas flowed out of the opercula, until midway through buccal compression. At peak buccal compression, a small amount of gas usually remained inside the mouth. Then, the buccal cavity expanded (as the mandible depressed) and filled with air. The fish closed its mandible, lowered its head below the water surface, and began compressing the buccal cavity, causing the gas bladder to inflate. As buccal compression finished, small bubbles of air leaked out of the buccal cavity (either at the dorsal ends of the opercular openings, at the ventral ends of the opercular openings (perhaps between the interopercula and the caudal attachment of the sternohyoid muscle on the cleithra), or at the rostral end of the jaws). This leakage of air occurred in most four-stroke breaths performed by Cb04, but none of the four-stroke breaths performed by Cb05. At the end of buccal compression, a small volume of air remained in the mouth. As gill ventilation resumed and the buccal cavity began expanding, the volume of gas inside the mouth expanded and the gas bladder deflated slightly, which was visible as a decrease in brightness in the gas bladder in the X-ray images. Buccal compression flushed this gas out of the mouth via the opercular openings.



VIDEO 1 X-ray video of Cb05 taking a four-stroke air breath. The video was recorded at 200 frames s^{-1} and slowed down 4 times. Video content can be viewed at https://onlinelibrary.wiley.com/doi/10.1002/ar.25460

3.3 | Inspiration-first breaths

The main phases of the inspiration-first breath are shown in Figure 3 (see Video 2). First, the fish approached the surface with its buccal cavity compressed. As the neurocranium and rostral ends of the jaws rose above the water surface, the buccal cavity expanded (as the mandible depressed) and filled with air. The fish closed its mandible, lowered its head below the water surface, and began compressing the buccal cavity, causing the gas bladder to inflate. Midway through buccal compression, bubbles of



VIDEO 2 X-ray video of Cb05 taking an inspiration-only air breath. The video was recorded at 200 frames s^{-1} and slowed down 4 times.

Video content can be viewed at https://onlinelibrary.wiley.com/doi/10.1002/ar.25460

air leaked out of the buccal cavity (either at the dorsal ends of the opercular openings, at the ventral ends of the opercular openings (perhaps between the interopercula and the caudal attachment of the sternohyoid muscle on the cleithra), or at the rostral end of the jaws). This leakage of air started earlier during buccal compression in inspiration-first breaths than in four-stroke breaths, and more air leaked out during inspiration-first breaths. This occurred in all inspiration-first breaths performed by both Cb04 and Cb05. At the end of buccal compression, a small volume of air remained in the mouth. As gill ventilation resumed and the buccal cavity began expanding, the volume of gas inside the mouth expanded and the gas bladder deflated slightly, which was visible as a decrease in brightness in the gas bladder in the X-ray images. Buccal compression flushed this gas out of the mouth via the opercular openings.

3.3.1 | Air-breathing behavior under aerial normoxia and hyperoxia

As in the X-ray videos (with no dome), in the standard videos with the breathing dome, the fish always performed a single breath and did not linger at the surface. Unlike in the X-ray videos, when the fish breathed in the dome, the fish almost always descended with a C-start-like motion (as described in *Hoplosternum littorale*, Domenici et al., 2015). Four-stroke and inspiration-first breaths were easily distinguished in the standard videos (Figure 3g, h).

Across Cb06, Cb07, Cb08, and Cb09, there were three instances when the fish expired air as it was approaching the surface, but it did not immediately perform the inspiration that would have completed the four-stroke breath. In two of those instances, this was because the fish had attempted to breathe outside of the breathing dome, and so could not reach the surface for inspiration. In the third instance, the fish had expired through both its mouth and its opercula, rather than only through the opercula, as is typical for this species. The fish seemed startled that it had done this and backed away from the surface instead of inspiring and completing the four-stroke breath. In all three instances, after expiring, the fish swam quickly around the tank and then performed an inspiration-first breath in the breathing dome between 9 and 15 s later. It appeared that these three pairs of expiration-only and inspiration-first breaths were intended to be four-stroke breaths. Because they were unusual, we excluded them from our data analysis.

Isolated expiration-only breaths were also observed, though, in nearly all cases, the volume of expired gas was small and occurred within 30 s after a breath, most often after an inspiration-first breath. These small expirations are not included in Figure 4a, but two somewhat larger expirations that were not taken soon after a previous breath are included.

The time sequences of breaths observed in each condition are shown in Figure 4a. At the start of the recording sessions, the mean (± s.d) partial pressure of oxygen in the water (P_{O2}) was 7.5 ± 0.9 kPa (specifically, oxygen concentration of 2.8 ± 0.4 mg/L at 26.3 ± 0.75°C), and at the end of the recording sessions, it was 5.2 ± 0.7 kPa (specifically, oxygen concentration of 2.0 ± 0.3 mg/L at 26.4 ± 0.75°C). Water saturated with air at atmospheric pressure has a P_{O2} of 21.3 kPa. Thus, these partial pressure values are 35% ± 4% and 24% ± 3% of the air-saturated values, respectively. Cb06, Cb07, and Cb08 took more total breaths in the aerial normoxia/aquatic hypoxia condition (41 ± 19) than in the aerial hyperoxia/aquatic hypoxia condition (16 ± 3). However, Cb09 did not take any breaths in the aerial normoxia/aquatic hypoxia condition. The percentage of inspiration-first breaths was higher in aerial hyperoxia/aquatic hypoxia (72% ± 40%, $n = 4$ individuals) compared to aerial normoxia/aquatic hypoxia (19% ± 15%, $n = 3$ individuals, Figure 4b). However, the data from Cb07 did not follow this pattern.

Cb07 took the same proportion of inspiration-first and four-stroke breaths in both experimental conditions (Figure 4). The percentage of inspiration-first breaths that Cb07 took in aerial normoxia/aquatic hypoxia (15%) was within the range observed in Cb06 (6%) and Cb08 (35%). In contrast, the percentage of inspiration-first breaths that Cb07 took in aerial hyperoxia/aquatic hypoxia (15.4%) was much smaller than those observed in Cb06 (72.2%), Cb08 (100%), and Cb09 (100%). In the aerial hyperoxia/aquatic

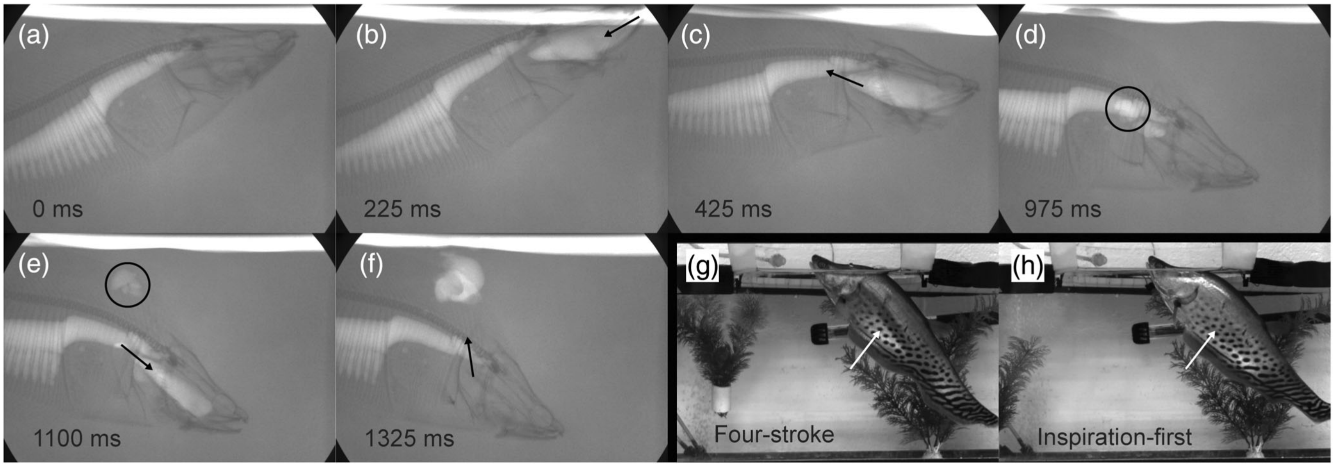


FIGURE 3 Frames from an X-ray video of *C. blanci* taking an inspiration-first breath, and frames from standard videos. During an inspiration-first breath, the fish (a) rises above the surface, (b) expands its mouth to take in air, (c) descends below the surface, (d) compresses its mouth and inspires air into the gas bladder, (e) expands its mouth as excess gas is expired from the gas bladder, and (f) compresses its mouth to expel gas out of the opercula. Air can be seen leaking out of opercula during buccal compression in (d) and (e), indicated with circles. Arrows indicate the direction of airflow. In standard videos, gas bladder deflation can be seen during the expiration portion of a four-stroke breath (g) but is not seen during an inspiration-first breath (h). White arrows indicate the location of the gas bladder.

hypoxia condition, Cb07 took fewer air breaths and the number of air breaths taken per hour decreased throughout the 6-h session. Cb07 also became increasingly negatively buoyant between breaths and reduced its swimming activity throughout the 6-h session. These observations suggest that Cb07 was avoiding taking air breaths and was not responding to the sensation of low buoyancy. This likely explains why Cb07 did not take many inspiration-first breaths in the aerial hyperoxia/aquatic hypoxia condition.

Cb09 only took inspiration-first breaths in the aerial hyperoxia/aquatic hypoxia condition, consistent with our hypothesis, but did not take any air breaths in the aerial normoxia/aquatic hypoxia condition. The fish approached the surface periodically but backed away without taking an air breath. It appeared likely that the fish did not feel safe or comfortable enough to take air breaths. Many air-breathing species are known to modify their air-breathing behavior in response to greater perceived risk of predation (Kramer & Graham, 1976; McKenzie et al., 2015; Smith & Kramer, 1986).

The binomial logistic mixed effects model found a significant effect of condition on the probability of an inspiration-first breath ($z = 5.645$, $p < 0.0000001$). The aerial hyperoxia/aquatic hypoxia condition had a logit of -2.8 ± 0.5, which translates to an odds ratio of 0.06. This means that there was a 94% decrease in the probability of a fish taking an inspiration-first breath when the condition was aerial normoxia/aquatic hypoxia instead of aerial hyperoxia/aquatic hypoxia. The magnitude of the random effects (i.e., individual) was 1.29 (standard deviation in log-odds scale). This translates to a standard deviation of the odds ratio of 3.63. This means that the probability of taking an inspiration-first breath varied among the individuals by up to 260%.

4 | DISCUSSION

4.1 | Function of four-stroke and inspiration-first breaths

The air-breathing behavior of *C. blanci* matches that of *Amia* when exposed to aerial normoxia and aerial hypoxia. In both species, the probability of taking inspiration-first breaths instead of four-stroke breaths was greater when the fish were breathing in 100% oxygen as compared to normoxic air ($p < 0.0000001$). Although Cb07 did not display this pattern, it also did not maintain neutral buoyancy. We believe that if it had maintained neutral buoyancy, it would have used the same behavioral response (inspiration-first breaths) that was used by the other individuals.

This indicates that in *C. blanci*, like in *Amia*, four-stroke breaths are stimulated by oxygen-sensitive chemoreceptors, whereas inspiration-first breaths are stimulated by stretch-sensitive mechanoreceptors in the gas bladder. While further testing the function of inspiration-first breaths in *C. blanci* is outside the scope of this paper, the modeling and experimental work conducted on *Amia* provide a strong theoretical foundation for this conclusion (Hedrick & Jones, 1999; Hedrick & Katz, 2016).

In *Amia*, Hedrick and Katz (2016) used mathematical modeling to test their hypothesis that four-stroke and

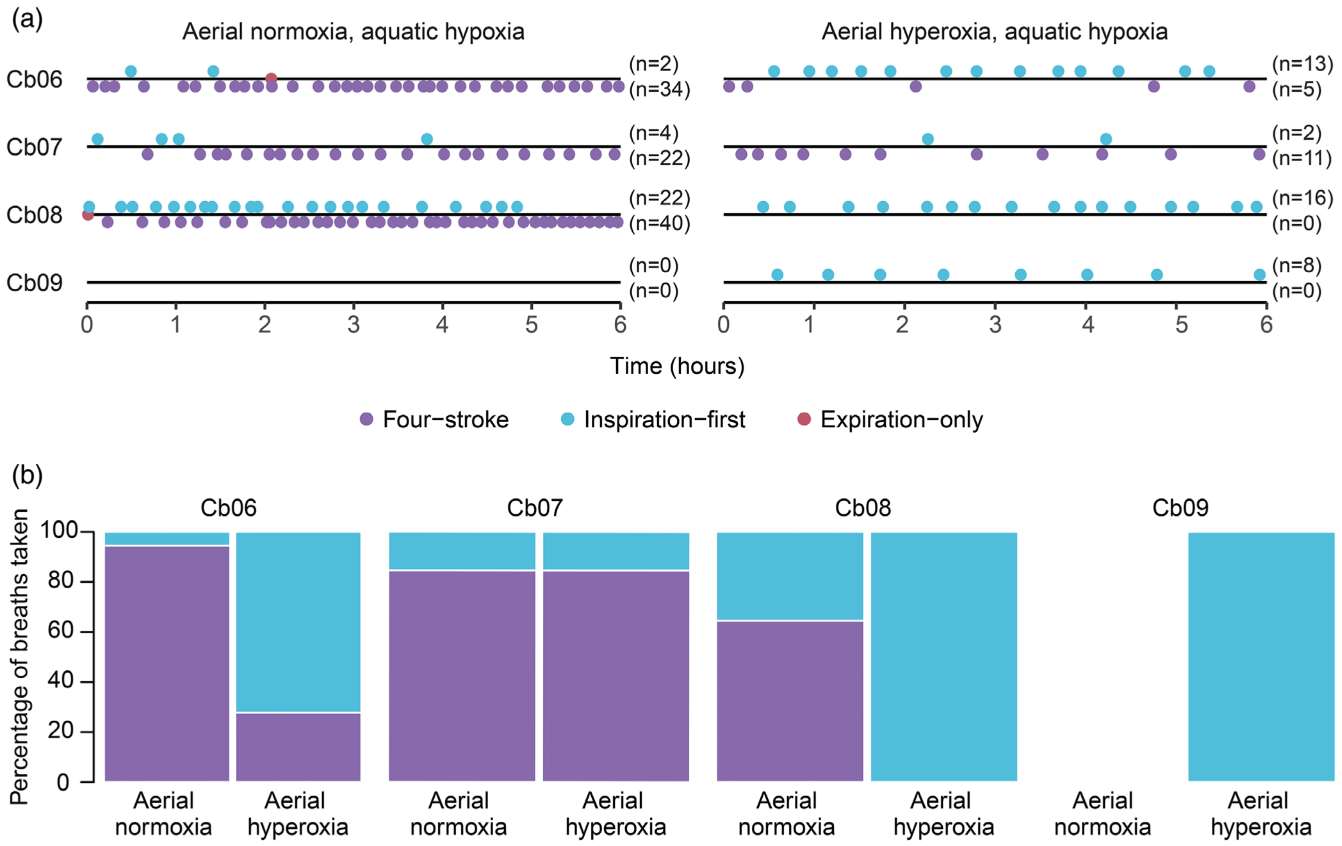


FIGURE 4 Air breaths taken in each experimental condition. (a) Each circle represents an air breath taken during the recording sessions (four-stroke breaths in purple, inspiration-first breaths in blue, and expiration-only breaths in orange). The data for the aerial normoxia/aquatic hypoxia condition is on the left, and the data for the aerial hyperoxia/aquatic hypoxia condition is on the right. The number of inspiration-first breaths (top) and the number of four-stroke breaths (bottom) taken by each individual are shown to the right of each timeline. (b) For each individual, the percentage of four-stroke breaths (purple) and inspiration-first breaths (blue) are shown for the aerial normoxia/aquatic hypoxia condition (left) and the aerial hyperoxia/aquatic hypoxia condition (right).

inspiration-first breaths are used in response to these separate physiological needs. In their model, low blood oxygen concentration triggered a four-stroke breath, and low ABO volume triggered an inspiration-first breath (with the expiratory and inspiratory volumes determined empirically). Because carbon dioxide is more readily lost at the gills than in the ABO in fish (Shelton et al., 2011), oxygen absorbed in the gas bladder is not replaced by carbon dioxide. So, after an air breath, the volume of the gas bladder and the oxygen concentration within it both decrease. As demonstrated by their mathematical model, the oxygen concentration of the inspired gas determines whether gas bladder volume or the blood oxygen concentration decreases faster. When inspiring 100% oxygen, gas bladder volume was depleted before oxygen was depleted, and so inspiration-first breaths were triggered most often. Compared to empirical observations of air-breathing in *Amia*, the mathematical model accurately predicted the increased use of inspiration-first breaths taken in aerial hyperoxia, the increased use of four-stroke breaths taken in aerial hypoxia, and the mean inter-breath interval of four-stroke breaths taken in aerial normoxia/aquatic hypoxia. These findings strongly supported their hypothesis that oxygen and buoyancy stimulate each breath type.

Hedrick and Jones (1999) also directly manipulated the gas bladder volume to test that this was the stimulus for inspiration-first breaths. When gas was experimentally removed from the gas bladder via a cannula, *Amia* responded by taking inspiration-first breaths, and when gas was added to the gas bladder, *Amia* responded by expiring gas. We believe that physiological mechanisms underlying the use of four-stroke and inspiration-first breaths in *Amia* are also at play in *C. blanci*.

4.2 | Airflow during inspiration-first breaths

In the inspiration portion of both the four-stroke breaths and the inspiration-first breaths, air leaked out of the buccal and/or opercular cavities during buccal compression, suggesting that the gas bladder became hyperinflated and could not take in any additional air. The air began leaking out of the buccal cavity earlier during inspiration-first breaths than it did for the inspiration portion of the four-stroke breath. This makes intuitive sense because in four-stroke breaths, gas is expired first, creating space in the gas bladder for more air to be inspired than in the inspiration-first breath.

In both breath types, as the buccal cavity reached peak compression and began to expand, some gas flowed back out of the gas bladder and then was expelled via the opercula with the subsequent buccal compression. This expiration of gas from the gas bladder was observed in all air breaths (including both four-stroke and inspiration-first breaths) in all individuals, in both the X-ray and standard videos. Based on the descriptions of air-breathing behaviors in *Amia* (Hedrick & Jones, 1993), it is not clear whether the same expiration of gas occurs at the end of inspiration in *Amia*.

The hyperinflation of the gas bladder and subsequent expiration of gas is intriguing because it creates the potential for inspiration-first breaths to be a sustainable source of oxygen. If a fish strictly inspires air, then these breaths would be very effective at maintaining buoyancy but would ultimately become very small and provide a negligible amount of oxygen. This is because, in lungs and gas bladders, oxygen is preferentially absorbed into the blood, leaving nitrogen gas behind. As oxygen is absorbed, the volume of the gas bladder decreases, stimulating another inspiration. This breath would replenish the gas bladder volume, but oxygen would only compose 21% of that inspired air. As nitrogen builds up in the gas bladder, the inspiratory volumes needed to restore buoyancy would become smaller, as would the oxygen provided. Therefore, some expiration of gas is needed in order to expel the nitrogen gas and make space for larger inspirations of air and oxygen. If the expiration of gas at the end of inspiration-first breaths were large enough, this could allow these breaths to provide a continued source of oxygen.

In fact, some air-breathing fishes appear to use such a breath. *Hoplerythrinus unitaeniatus*, *Piabucina festae*, and *Gymnotus carapo* are all described using a breath type that is very similar to the inspiration-first breath we observed in *C. blanci* (Graham et al., 1977; Kramer, 1978; Liem et al., 1984). The fish comes to the surface, expands its mouth to take in air, compresses the mouth to transfer air into the gas bladder (at which point bubbles sometimes escape out of the opercula in *H. unitaeniatus* and *P. festae*, as we observed in *C. blanci*), lowers below the surface and expands its mouth (with its jaws open), and upon compressing the mouth, a large bubble is expelled out of the operculum (Graham et al., 1977; Kramer, 1978; Liem et al., 1984). X-ray film taken of *H. unitaeniatus* and *G. carapo* revealed that the inspired air is fully transferred to the gas bladder (except for any bubbles that were lost through the opercula during buccal compression) before gas is then released from the gas bladder and expelled out of the mouth via the opercula (Kramer, 1978; Liem et al., 1984). In all three species, the authors interpreted these observations as indicating that the gas bladder was overfilled and that excess gas was then expired from the gas bladder (Graham et al., 1977; Kramer, 1978; Liem et al., 1984). This matches the pattern of airflow and buccal pumping that we observed in *C. blanci*.

Keeping the gas bladder pressurized may prevent the gas bladder from changing volume as much between breaths, which may be advantageous to species that use their gas bladder for sound detection, including *H. unitaeniatus*, *P. festae*, and *G. carapo*, as well as *C. blanci*. It is also possible that hyperinflation of the gas bladder does not provide a functional benefit for sound detection and is simply an intermediate stage between inspiring and expiring gas.

The air-breathing behaviors of two other fish, *Misgurnus angullicaudatus* and *Pantodon buchholzi*, also begin with an inspiration of air followed by a release of bubbles, but in *P. buchholzi* it is not clear whether the gas bladder is hyperinflated and then expires air, and in *M. anguillicaudatus* the ABO is the digestive tract, and so bubbles are released out of the vent, not the opercula (McMahon & Burggren, 1987; Schwartz, 1969).

While the breath types used by these species are similar to the inspiration-first breaths described in *C. blanci*, there is an important difference: the air breaths used by these species are sensitive to oxygen availability (Graham et al., 1977; Liem et al., 1984; McMahon & Burggren, 1987; Schwartz, 1969). This implies that these air breaths provide a sustainable source of oxygen for these species, permitted by expiration of sufficient volume of gas. It is not known how the tidal volumes in these species compare to that of the inspiration-first breath in *C. blanci*. Nor is it known whether these species would also use this type of air breath to restore buoyancy.

4.3 | Terminology for air-breath types

Because gas was expired after inspiration, we have decided to use the terminology "inspiration-first breaths." Note that "inspiration" signifies movement of air into an ABO, not simply intake of air into the mouth. Conversely, "expiration" signifies movement of air out of the ABO, not simply expulsion of gas from the mouth. The term "inspiration-first" is intended to emphasize that the breath begins with inspiration of air into the ABO but not specify whether expiration occurs afterwards (as observed in *C. blanci*). Future research may find that species vary in whether gas is expired from the gas bladder after inspiration and in how much gas is expired. The term 'inspiration-first' is also intended to distinguish this breath type from four-stroke and two-stroke breaths, in which expiration precedes inspiration, and in which the expiration volume is generally quite large (e.g., 15%–33% of gas bladder volume in *Amia* (Hedrick & Jones, 1993), and seemingly close to 100% of lung volume in lungfish (Bishop & Foxon, 1968; Kaczmarek et al., 2022; McMahon, 1969)).

In *C. blanci*, release of gas from the gas bladder (expiration) occurs after inspiration in both four-stroke and inspiration-first breaths. So, in this species, four-stroke breaths involve expiration, then inspiration, and then a second expiration of gas. This second expiration is smaller than the first, contains a mixture of newly inspired and previously inspired gas, and may not be common to the four-stroke breaths of other species. To avoid confusion, it should be assumed that, in this paper, discussion of the expiration portion of a four-stroke breath refers to the initial expiration of gas, unless clearly specified otherwise.

We also want to emphasize that although inspiration-first breaths involve two "strokes" (one buccal expansion and one buccal compression), they do not fall within the category of "two-stroke" breaths. The term "two-stroke" breath should be reserved for the type of breath that is commonly used among sarcopterygians that breathe air using buccal pumping, and that is therefore hypothesized to be ancestral to this lineage. This two-stroke breath describes a particular sequence of buccal motion and airflow that does not match the sequence observed in inspiration-first breaths. In two-stroke breaths, the animal first comes to the surface and draws air into its mouth, then expires air from the lungs out through the open mouth, and then compresses its mouth to pump new air into the lungs (Figure 1a). Similarly, the term "four-stroke" breath should not be used to refer to any breath that uses four "strokes" and should instead be reserved for the type of breath that is commonly used among actinopterygians and is hypothesized to be ancestral to this lineage (Figure 1b).

4.4 | Mechanism of expiration in *C. blanci*

The mechanism of expiration during four-stroke breaths in *C. blanci* is likely hydrostatic pressure on the body wall, with possible contributions from striated muscle fibers within the gas bladder wall (Dehadrai, 1962) and elasticity of the gas bladder wall. The expiration of gas from the gas bladder that occurred immediately after inspiration in *C. blanci* (in both inspiration-first breaths and four-stroke breaths) was likely caused by a combination of the elasticity of the gas bladder (which appeared overfilled) and hydrostatic pressure on the body wall, with striated muscle activity being possible but not necessary. While it is known that muscle fibers within the lung walls of polypterid fishes cause expiration (Brainerd, 1994b; Brainerd et al., 1989), this has not been studied in other air-breathing fishes.

4.5 | Prevalence of inspiration-first breaths

In *C. blanci*, like in *Amia*, four-stroke breaths are stimulated by a need for oxygen, and inspiration-first breaths are stimulated by a need for buoyancy. We believe other air-breathing species likely use inspiration-first breaths to increase buoyancy as well, especially species that have been observed using four-stroke or two-stroke breaths.

One reason we believe this is that researchers have most often elicited air-breathing behaviors by reducing aquatic oxygen. This is an effective way to stimulate air breaths, but it may have masked the use of air breaths to regulate buoyancy. When exposed to aerial normoxia and aquatic hypoxia, both *C. blanci* and *Amia* predominately used four-stroke breaths. And so, the four-stroke breaths of both species had been studied in detail without observing their use of inspiration-first breaths (Deyst & Liem, 1985; Liem, 1988; Liem, 1989; Randall et al., 1981). It is possible that the same oversight has occurred for other air-breathing species.

To reveal how air-breathing behavior is modulated to regulate buoyancy, buoyancy must be manipulated as a stimulus for air breathing. Probing the role of air breaths for buoyancy regulation can be done by directly manipulating the buoyancy of the fish, or by using exposure to aerial hyperoxia to disentangle the need for oxygen from the need for buoyancy, as was done by Hedrick and Jones (1993) and in this study. Given the lack of such experiments, the scarcity of inspiration-first and expiration-only breaths in the literature does not indicate that air-breathing fishes do not perform them.

We think there is particular reason to believe that lungfish may use inspiration-first breaths to regulate buoyancy. As expected for sarcopterygian species that breathe air using buccal pumping, lungfish use two-stroke breaths, which involve both expiration and inspiration (Bishop & Foxon, 1968; Brainerd et al., 1993; Kaczmarek et al., 2022; McMahon, 1969). Interestingly, this two-stroke breath is often immediately followed by one to four additional inspirations of air. This has been observed in *Lepidosiren* paradoxa (Bishop & Foxon, 1968), *Protopterus aethiopicus* (McMahon, 1969), and *Neoceratodus forsteri* (E.B.K. personal observation). During the two-stroke breath, lungfish typically expire until their lungs are nearly empty (Bishop & Foxon, 1968; Kaczmarek et al., 2022; McMahon, 1969). The additional inspirations are hypothesized to be necessary to fully reinflate the lungs—one mouthful of inspired air is typically not enough. When McMahon (1969) cannulated the lungs of *P. aethiopicus* and kept the lungs open to atmospheric air, the fish took repeated inspirations and stayed at the surface. In contrast, *P. aethiopicus* exposed to aerial anoxia took a single inspiration and then stayed at the bottom. This indicates that additional inspirations are stimulated by insufficient lung volume, rather than insufficient oxygen (McMahon, 1969). Additionally, lungfish have pulmonary mechanoreceptors that fire faster as lung volume increases (Delaney et al., 1983). These lines of evidence suggest that lungfish may use inspiration-first breaths to regulate buoyancy.

4.6 | Evolution of inspiration-first breaths and the origin of air-breathing

If inspiration-first breaths are indeed common in air-breathing fish, as suggested by our results from *C. blanci*, it would lay the foundation for a new perspective on the origin of air-breathing behaviors in Osteichthyes. We discuss prior literature on this subject and present our hypothesis for the origin of air-breathing.

There is strong evidence that air-breathing anatomy (lungs) evolved once in the ancestors of Osteichthyes (Cass et al., 2013; Funk, Breen, et al., 2020; Funk, Lencer, & McCune, 2020; Liem, 1988; Longo et al., 2013), but the early evolutionary history of air-breathing behavior is less clear. Four-stroke and two-stroke breaths are hypothesized to be ancestral to Actinopterygii and Sarcopterygii, respectively, but that dichotomy makes it difficult to theorize about how these two breath types originated (Brainerd, 1994a). As enumerated by Brainerd et al. (1993), there are three possible hypotheses: (1) four-stroke and two-stroke breaths originated independently, (2) one of these breath types is derived from the other, and (3) they are both derived from a third unknown breath type present in the common ancestor of Osteichthyes.

Two studies have proposed the independent origins of four-stroke and two-stroke breaths (Brainerd, 1994a; Perry et al., 2001). Since the publication of those papers, more evidence has been found supporting the homology of lungs and gas bladders (Cass et al., 2013; Funk, Breen, et al., 2020; Funk, Lencer, & McCune, 2020; Longo et al., 2013). While the hypothesis proposed by Perry et al. (2001) attributes any similarities between the lungs of Polypteriformes, the gas bladders of Actinopteri, and the lungs of Sarcopterygii to their common derivation from the posterior pharynx, this hypothesis depends heavily on shared developmental precursors to yield very similar outcomes in different clades. Instead, we believe that the evidence for homology of lungs and gas bladders reflects their shared ancestry and the single origin of lungs in the common ancestor of Osteichthyes. We presume that lungs and an associated ventilatory behavior evolved alongside each other, so growing evidence for a single origin of lungs makes hypotheses for the independent evolutions of four-stroke and two-stroke breaths less likely.

Much theorizing about the origin of air-breathing behaviors has sought to identify which of the aquatic buccal pumping behaviors (e.g., gill ventilation, suction feeding, coughing) air-breathing might have evolved from (Brainerd, 1994a; McMahon, 1969; Perry et al., 2001; Smatresk, 1990). However, it is unclear whether this line of reasoning is useful. This is in part because it is not intuitive that air breaths would be derived from a preexisting complex, coordinated, and regulated behavior. In addition, this is in part because evidence for a single origin of lungs and a single ancestral breath type suggests four-stroke and two-stroke breaths are derived from each other or from a third ancestral breath type.

We hypothesize that inspiration-first breaths were the original breath type used by air-breathing fishes and that four-stroke and two-stroke breaths were derived by combining inspiration-first and expiration-only breaths in two different ways (Figure 5). Inspiration-first breaths are perhaps the simplest breath type necessary for maintaining buoyancy as gas diffuses out of highly permeable lungs. For inspiration-first breaths to also be effective as a source of oxygen, some expiration of gas is necessary to remove the nitrogen that accumulates as oxygen is absorbed in order to make space for new oxygen-rich air to be inspired. Expiration-only breaths may have evolved alongside, or after, inspiration-first breaths. We hypothesize that four-stroke and two-stroke breaths then evolved as the combination of inspiration-first and expiration-only breaths, producing two distinct, stereotyped behaviors. Four-stroke breaths are easily conceived of as an expiration-only breath followed by an inspiration-first breath, whereas two-stroke breaths can be conceived of as an inspiration-first breath with an expiration performed in the middle (the buccal cavity expands at the surface, taking in fresh air, the lungs deflate, and then the buccal cavity compresses, causing the lungs to inflate). This hypothesis is similar to that put forward by Hedrick and Katz (2016), who also proposed that inspiration-first breaths evolved first, before four-stroke

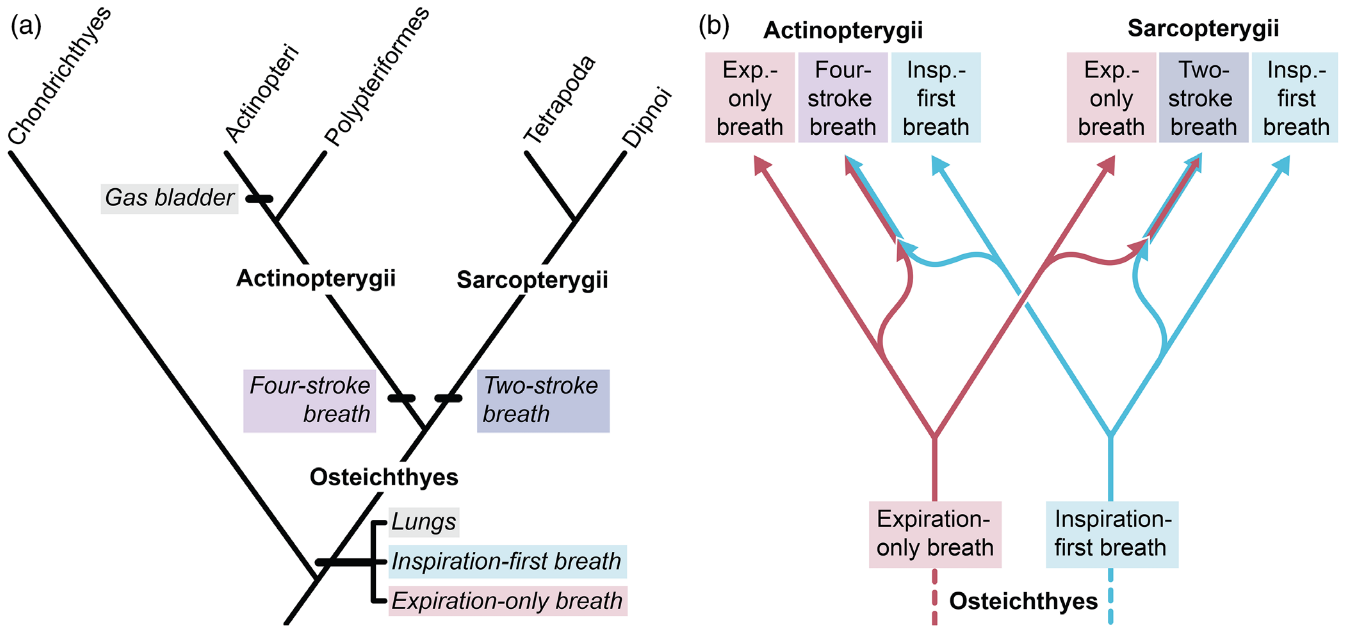


FIGURE 5 Hypothesized evolutionary origins of and relationships between air-breath types. (a) Cladogram illustrating the hypothesized sequence of origins of air-breath types. We hypothesize that inspiration-first and expiration-only breaths originated at the base of Osteichthyes, as the original air-breathing behaviors. Phylogenetic relationships adapted from Betancur-R et al. (2017). (b) Diagram illustrating the hypothesized evolutionary relationships between air-breath types. We hypothesize that the original air breath types used by Osteichthyes were the inspiration-first and expiration-only breaths. We also hypothesize that two-stroke and four-stroke breaths later evolved as a combination of inspiration-first and expiration-only breaths, producing two distinct, stereotyped behaviors in Sarcopterygii and Actinopterygii. In parallel, inspiration-first breaths and expiration-only breaths continue to be used by air-breathing fishes.

breaths, but did not address the evolution of the expiration component, nor articulate the relationship between inspiration-first, four-stroke, and two-stroke breaths. The focus of their hypothesis was that buoyancy may have been the primary selective force driving the origin of air-breathing.

Although the widespread use of inspiration-first breaths (by air-breathing fishes that also use four-stroke and two-stroke breaths) would be consistent with the hypothesis that inspiration-first breaths are ancestral to Osteichthyes, we acknowledge that it would not provide sufficient support for our proposed scenario that four-stroke and two-stroke breaths are derived from an ancestral inspiration-first breath. Future work on the neural control and circuitry of air-breathing behaviors may shed light on this hypothesis.

AUTHOR CONTRIBUTIONS

Elska B. Kaczmarek: Conceptualization; formal analysis; investigation; methodology; writing – original draft. **Elizabeth L. Brainerd:** Resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

X-ray and standard video data and their essential metadata are stored in the XMAPortal (xmaportal.org) in the study “Knifefish Aerial and Aquatic Breathing” with the permanent identifier BROWN79. Examples of the video data are publicly available under CCBY 4.0 in the public data collection “Air breathing videos for Kaczmarek and Brainerd, 2024” on XMAPortal: https://xmaportal.org/webportal/larequest.php?request=CollectionView&StudyID=79&instit=BROWN&collectionID=26.

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ENDNOTE

[1] Note that in the original research on these breath types in *Amia*, the authors refer to four-stroke breaths as Type I and inspiration-first breaths as Type II breaths and do not provide a name for isolated expirations of air (Hedrick & Jones, 1993).

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