RESEARCH ARTICLE

The mechanics of air breathing in African clawed frog tadpoles, *Xenopus laevis* (Anura: Pipidae)

Jackson R. Phillips*,§, Amanda E. Hewes‡, Molly C. Womack* and Kurt Schwenk

ABSTRACT

Frog larvae (tadpoles) undergo many physiological, morphological and behavioral transformations throughout development before metamorphosing into their adult form. The surface tension of water prevents small tadpoles from breaching the surface to breathe air (including those of *Xenopus laevis*), forcing them to acquire air using a form of breathing called bubble sucking. With growth, tadpoles typically make a behavioral/biomechanical transition from bubble sucking to breaching. *Xenopus laevis* tadpoles have also been shown to transition physiologically from conforming passively to ambient oxygen levels to actively regulating their blood oxygen. However, it is unknown whether these mechanical and physiological breathing transitions are temporally or functionally linked, or how both transitions relate to lung maturation and gas exchange competency. If these transitions are linked, it could mean that one biomechanical breathing mode (breaching) is more physiologically proficient at acquiring gaseous oxygen than the other. Here, we describe the mechanics and development of air breathing and the ontogeny of lung morphology in *X. laevis* throughout the larval stage and examine our findings considering previous physiological work. We found that the transitions from bubble sucking to breaching and from oxygen conforming to oxygen regulation co-occur in *X. laevis* tadpoles at the same larval stage (Nieuwkoop–Faber stages 53–56 and 54–57, respectively), but that the lungs do not increase significantly in vascularization until metamorphosis, suggesting that lung maturation, alone, is not sufficient to account for increased pulmonary capacity earlier in development. Although breach breathing may confer a respiratory advantage, we remain unaware of a mechanistic explanation to account for this possibility. At present, the transition from bubble sucking to breaching appears simply to be a consequence of growth. Finally, we consider our results in the context of comparative air-breathing mechanics across vertebrates.

KEY WORDS: Anura, Functional morphology, Physiology, Behavior, Respiration, Lungs, Development

INTRODUCTION

Vertebrate lungs and other air-filled organs serve a diverse range of functions across different lineages and life stages (Carrier, 1987; Liem, 1988; Graham, 1997; Brainerd, 1999; Graham and Wegner,

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2010; Hsia et al., 2013). For example, air breathing can serve purely for gas exchange in mammalian lungs (Schmidt-Nielsen, 1997), for hydrostatic control in the gas bladders of physostomous fishes (Scholander, 1956; Alexander, 1990; Uotani et al., 2000; Smith and Croll, 2011), or for both gas exchange and hydrostatic control in the gas bladders of air-breathing physostomous fishes (Graham, 1997; Hedrick and Jones, 1999). In amphibians that retain a biphasic lifestyle, the biological role of air breathing can differ substantially between larvae and adults. For most anurans (frogs and toads), the lungs are important both for buoyancy (hydrostatic) control and gas exchange in aquatic larvae (tadpoles), while the hydrostatic function is lost in most adults (Wassersug and Seibert, 1975; Burggren and West, 1982; Burggren and Mwalukoma, 1983; Gee and Waldick, 1995; Gee and Rondeau, 2012). The function of the lungs also shifts within the tadpole phase, from a nearly exclusive hydrostatic role after hatching to a much greater role in gas exchange later in development (Burggren and West, 1982; Hastings and Burggren, 1995; Phillips et al., 2020). The degree to which the lungs function in gas exchange may be reflected both in the biomechanics of air breathing and in the morphology of the lungs (Phillips et al., 2020).

Tadpole air breathing has been studied both physiologically (e.g. Burggren and West, 1982; Hastings and Burggren, 1995) and biomechanically (Wassersug and Yamashita, 2000; Schwenk and Phillips, 2020; Phillips et al., 2020), but there has been little integration of these disciplines. The African clawed frog, *Xenopus laevis*, is perhaps the species whose larval air-breathing physiology is best understood, making it an excellent candidate for cross-disciplinary study. *Xenopus laevis* tadpoles are midwater suspension feeders with an unusual head-down swimming posture and derived extensions of the lungs called 'dorsal diverticulae', which are believed to function hydrostatically to maintain this posture (Bles, 1906; Weisz, 1945a,b; Van Bergeijk, 1959; Fejtek et al., 1998). The lungs also play an important role in larval *X. laevis* respiration – air-breathing rates increase as oxygen partial pressure decreases or the branchial (gill) surfaces become covered with food (Bles, 1906; Feder and Wassersug, 1984; Feder et al., 1984). However, Hastings and Burggren (1995) found that only late-stage *X. laevis* larvae were able to extract significant oxygen via air breathing. They showed that at Nieuwkoop–Faber (NF) stages 54–57 (Nieuwkoop and Faber, 1994), *X. laevis* tadpoles transition from having blood oxygen determined passively by ambient oxygen levels (oxygen conforming) to actively regulating their blood oxygen to concentrations above ambient levels (oxygen regulating). They speculated that this transition was a result of lung maturation, and that older tadpoles were able to survive hypoxia by supplementing cutaneous and branchial respiration with air breathing. Hastings and Burggren (1995) suggested that *X. laevis* tadpoles transition to oxygen regulation because of a shift from non-respiratory to respiratory air breathing at ~NF stages 54–57. Notably, however, those authors did not examine lung development in detail.

Although the transition from oxygen conformation to oxygen regulation occurs midway through the larval development of *X. laevis*, much smaller, younger tadpoles are also known to air breathe (Bles, 1906; Ultsch et al., 1999). Schwenk and Phillips (2020) found that these smaller individuals performed a previously undescribed form of air breathing they called 'bubble sucking', while larger individuals breached the surface to breathe (as described by Wassersug and Yamashita, 2000), as is typical of most aquatic vertebrates (Fig. 1). Bubble sucking is a form of air breathing in which a tadpole (or other small animal) attaches to the underside of the water's surface tension and sucks the air–water interface down into the mouth, capturing a bubble, which is then compressed into the lungs. It is unknown whether bubble sucking plays a respiratory role at any point in *X. laevis* tadpoles or whether the transition from bubble sucking to breach breathing corresponds to the transition from oxygen conformation to oxygen regulation.

Schwenk and Phillips (2020) suggested that bubble sucking circumvents a physical constraint on breaching in individuals too small or slow to break through the water's surface tension. This hypothesis is supported by first principles (i.e. the underlying physical properties of surface tension), frequent instances of small tadpoles attempting, but failing, to break through the water surface, and the observation that the transition from bubble sucking to breaching in tadpoles of several species occurs at approximately the same body size (K.S. and J.R.P., unpublished data). The last observation, in particular, suggests that the transition point to breach breathing is determined by an interaction between the physical properties of surface tension and a tadpole's phenotype (body size, head shape, swimming speed, etc.). This implies that there is a lower limit on body size or developmental stage before which a tadpole cannot breach to breathe. However, there is no apparent upper limit on size or stage for bubble sucking prior to metamorphosis. Indeed, tadpoles of most species continue to bubble suck occasionally, or even frequently, after they begin to breach breathe and at least one species (*Hyla versicolor*) never transitions to breaching at all, continuing to bubble suck throughout larval ontogeny (Phillips et al., 2020).

Why, then, do most tadpoles transition to breaching once they are physically able? The null hypothesis is that bubble sucking and

breach breathing are functionally and energetically equivalent and that the transition is merely a consequence of growth and the attainment of sufficient mass and velocity. However, the transition might reflect that tadpoles somehow benefit from breach breathing or that bubble sucking incurs a cost. For example, breaching may require less energy than deforming the water's surface to bubble suck; breaching might capture a greater volume of air, or it could be faster, minimizing time spent at the surface vulnerable to predators. To elucidate the proximate and ultimate determinants of these processes, the mechanical and functional differences between bubble sucking and breaching must be better understood.

In a previous study, Phillips et al. (2020) showed that a transition in the biomechanics of air breathing was tightly correlated with lung development and a likely increase in pulmonary gas-exchange capacity. They found that *Hyla versicolor* tadpoles switched to a novel air-breathing mode at the same point in ontogeny that their lungs developed significant pulmonary vasculature. The *H. versicolor* tadpoles never transitioned to breach breathing, but instead shifted midway through the larval period from the typical (ancestral) form of bubble sucking ('single bubble sucking') to a derived pattern in which a second suction event immediately follows the first ('double bubble sucking'). Double bubble sucking separates lung emptying from lung filling and prevents the mixing of freshly inspired air with residual air from the lungs, which should increase the efficiency of gas exchange by preventing the consequent decrease in pulmonary oxygen concentration. This shift in bubble-sucking mechanics is mirrored precisely by a sudden increase in lung vascularization. These concordant changes suggest that air breathing in *H. versicolor* changes during the larval period from a non-respiratory to a respiratory function (Phillips et al., 2020). The study was limited, however, by a lack of direct physiological data for air breathing, so function could only be inferred from lung morphology and breathing mechanics.

Like *H. versicolor*, *X. laevis* tadpoles have also been suggested to undergo an ontogenetic transition in the gas exchange competency of air breathing. Hastings and Burggren (1995) demonstrated physiologically that *X. laevis* tadpoles transition from oxygen conforming to oxygen regulating. Subsequently, Schwenk and Phillips (2020) showed that *X. laevis* tadpoles also undergo a mechanical transition in breathing mode, from single bubble sucking to breach breathing, midway through the tadpole phase. We do not know, however, whether these mechanical and physiological transitions are coincident or functionally related. If they are coincident, it might be because breaching provides an advantage for gas exchange compared with bubble sucking. If this is the case, breach breathing could be part of an adaptive suite of coordinated developmental changes that facilitate gas exchange during later larval life and we would expect to find phenotypic differences between bubble sucking and breaching that are consistent with this, such as a rapid increase in pulmonary vasculature, as we observed in *H. versicolor* tadpoles. Pulmonary vasculature facilitates transport of oxygen to areas not directly adjacent to the lungs, which might become essential as body size increases. It also serves to maintain a favorable concentration gradient to increase the rate of gas exchange. We therefore assume that a rapid increase in pulmonary vasculature would lead to an increase in the capacity for pulmonary gas exchange (note, however, that direct physiological measurements of performance would be necessary to demonstrate that this increase in the potential for gas exchange results in a significant functional advantage). Comparative evidence across adult anurans supports this assumption. Stream-dwelling forms that rely principally on
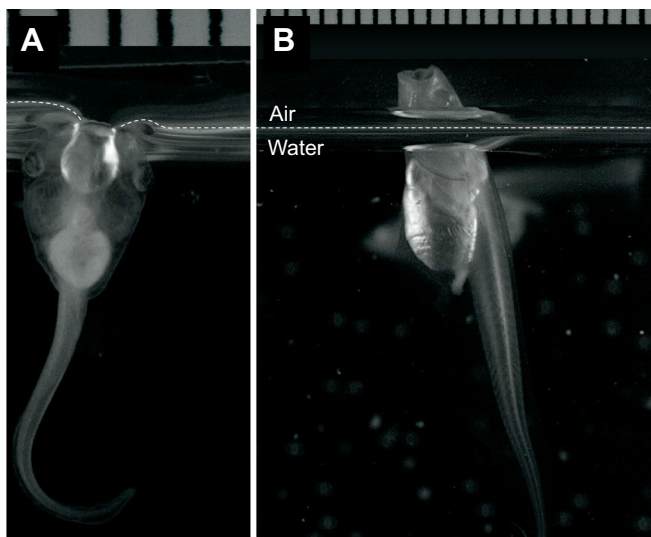

Fig. 1. Tadpole breathing modes. (A) A young *Xenopus laevis* tadpole draws the surface of the water down into the mouth to perform a bubble suck. (B) An older and larger *X. laevis* tadpole breaks through the surface tension to breach breathe. In both A and B, each tick mark is 1 mm (top of images).

cutaneous respiration have far less vascular lungs than their terrestrial counterparts (Noble, 1925, 1931).

In this study, we observed air breathing from hatching to metamorphosis in tadpoles of *X. laevis*. Our primary goals were to quantify the kinematics of air breathing over time and to relate them to published transitions in air-breathing physiology. We sought to answer several questions in relation to those goals. (1) Do the mechanics of bubble sucking and breaching differ from one another? Is there any evidence that one is more advantageous for gas exchange than the other? (2) As tadpoles grow and mature, are there any changes in air-breathing behavior unrelated to the bubble-sucking to breach transition? For example, do the breaths of smaller bubble suckers differ kinematically from those of larger bubble suckers? (3) What is the temporal relationship between the bubble-sucking to breach transition and the known physiological transition from oxygen conforming to regulating? (4) What is the relationship between lung development and both transitions?

The evolution of air-breathing behavior across vertebrates is shaped not only by the divergent needs of different species and lineages but also by changing needs and functions over the course of ontogeny. There have been few previous studies that focused on how air-breathing biology can change across developmental transitions. Our study seeks to illuminate the relationships between physiological strategies for gas exchange, lung morphology and air-breathing biomechanics that characterize a key ontogenetic transition in *X. laevis* tadpole respiration.

MATERIALS AND METHODS
Animals
We purchased 100 fertilized eggs of albino African clawed frogs (*Xenopus laevis* Daudin 1802) from a commercial dealer (Nasco, Fort Atkinson, WI, USA). Eggs were maintained in 38 l aquaria filled with 19 l of de-chlorinated tap water kept at room temperature (20°C). Each aquarium held multiple clutches of eggs. Tadpoles were distributed to additional aquaria to maintain approximately 50–200 tadpoles per aquarium, depending on size. They were fed commercial food (Nasco 'frog brittle'^{TM}) every 2 days. Aquaria were regularly cleaned with hot water and soap. We used albino tadpoles to visualize the movements of air in to and out of the lungs with simple light videography.

For anatomical studies, we killed tadpoles via anesthetic overdose using a bath of 10% benzocaine ointment dissolved in water (AVMA, 2013). We then rinsed tadpole specimens in deionized water and either dissected them immediately under a dissecting microscope or fixed specimens in 10% formalin solution for at least 1 week before transferring them into 70% ethanol for long-term storage or histology. All live animal use was approved by the University of Connecticut Institutional Animal Care and Use Committee (IACUC protocol A18-032).

High-speed videography
We used an Edgertronic SC1 monochrome camera with a Nikon 105 mm, f/2.8 macro lens to obtain close-up, high-speed video of tadpoles air breathing. During filming, tadpoles were placed in a small, glass or plastic chamber that allowed unrestricted motion. A millimeter scale was placed within the field of view of the video frame on the inner surface of the filming chamber's front pane. Illumination was provided by three banks of continuous (video) LED lights (500 LED lights, ikan®, Houston, TX, USA), plus supplemental lighting as needed. Videos were taken at 300–1000 frames s^{-1}.

Air-breathing sequences were analyzed and quantified using the freeware program Tracker® v.4.11.0 (https://physlets.org/tracker/).

The program was calibrated using the millimeter scale within the frame of each video so that tadpole snout–vent length (SVL) could be measured. Each breathing bout was scored as either a breach if the snout protruded above the surface, breaking the surface tension, or a bubble suck if the mouth attached and sucked down a bubble from the surface.

Phillips et al. (2020) identified several kinematic events that occur during a complete bubble-sucking breath: attachment, lung emptying, pinch off, lung filling and bubble release. Although homologous events occur during breach breathing, some of the bubble-sucking nomenclature is inappropriate. The equivalent breathing events or stages for breaching are: attachment=mouth open and pinch off=mouth close. The phase of air breathing from attachment to pinch off or mouth open to mouth close will be referred to as 'suction' for both bubble sucking and breaching, as in both cases air is drawn into the mouth via negative pressure. Following air acquisition, the remaining kinematic phases of bubble sucking and breaching are largely indistinguishable, and we use the same terminology.

Designating 'attachment' (or mouth open) as time zero, we used Tracker® to calculate the duration of each kinematic phase in seconds for all breathing bouts. We measured duration for the following kinematic phases: 'suction' (time from attachment to pinch off), 'compression' (time from pinch off to bubble release), 'suction I' (time from attachment/mouth open to the beginning of lung emptying), 'lung emptying' (self-evident), 'suction II' (time from the end of lung emptying to pinch off), and lung filling (self-evident). Individuals filmed breathing were also staged according to NF stage (Nieuwkoop and Faber, 1994) from the video. We collected a total of 158 usable videos, five of which included multiple breaths from different individuals. In total, we analyzed 164 breaths (Table 1; Table S3). In some cases, not all kinematic phases were measurable in a given video (e.g. if a tadpole swam out of frame before releasing a bubble following compression).

Paraffin histology
We prepared 10 individuals, ranging in size from 2.7 to 13.7 mm SVL, for paraffin histology using standard methods (Presnell and Schreibman, 1997). We embedded specimens in 100% paraffin and sectioned them in the frontal plane at 6–10 µm to examine lung structure and vascularization. Sections were then stained with Weigert iron hematoxylin and picro-ponceau (Presnell and Schreibman, 1997).

Quantitative analyses
Statistical analyses
All analyses were performed with R version 3.6.2 (http://www.R-project.org/). Unless otherwise specified, we natural log transformed our duration data in order to meet model assumptions.

Table 1. Frequency of different breathing modes and breathing stages observed in *Xenopus laevis* tadpoles

Breathing mode/stage	No. of individuals	% Total
Bubble sucks (all)	97	59.15
Breaches (all)	67	40.85
Early bubble sucks	56	34.15
Late bubble sucks	41	25.00
Pre-metamorphic breaches	49	29.88
Metamorphic breaches	18	10.97

Choosing the best predictor of development

We measured several potential proxies of development for our analyses: body size (SVL), age (in days from hatching) and NF stage. Previous studies on the development of air-breathing mechanics have relied primarily on SVL as a proxy for development (e.g. Phillips et al., 2020). However, *X. laevis* tadpoles reach a maximum body length (SVL) partway through ontogeny, and then decrease in length through metamorphic climax. We performed a breakpoints analysis with the R package 'segmented' v.1.1-0 (Muggeo, 2008) to test whether SVL was a good predictor of development (Fig. 2). This analysis regresses SVL over NF stage, and then allows two independent lines to fit the data, with a 'breakpoint' separating the lines. Using an AIC comparison, we found the breakpoints model fitted our data significantly better than a simple linear regression (simple$_{AIC}$=641; breakpoints$_{AIC}$=474). This showed that SVL alone is not a suitable proxy of development for *X. laevis* tadpoles.

Age was also a problematic predictor of development, as tadpoles developed at widely divergent rates, with some of our oldest individuals never approaching metamorphosis. Developmental stage was our most reliable proxy for true development, but at least one stage (NF 47) included individuals of substantially different SVL, indicating that considerable growth occurred during this stage. Because no single measure of development sufficiently captured the resolution of development we desired, we used linear modeling to test the effects of SVL within discrete developmental breathing stages (see below) on the duration of the kinematic phases of air breathing over ontogeny.

Determining the biomechanical transition point from bubble sucking to breaching

We modeled the transition from bubble sucking to breaching using two binomial generalized linear models (GLMs) of breathing mode, one as a function of SVL and the other as a function of NF stage. We then used the generated model parameters to estimate the point at which the probability of breaching is 50%, which we took to be the transition point from bubble sucking to breaching.

Comparing the kinematics of bubble sucking versus breaching

We compared bubble sucking and breaching by the durations of the kinematic phases of air breathing (for a full list of the kinematic phases measured, see 'High-speed videography', above). We used Welsh's two-sample *t*-tests to evaluate whether the duration of each phase differed significantly between bubble sucking and breaching.

Modeling duration of kinematic phases across development

We also examined the duration of kinematic phases across ontogeny, accounting for breathing mode, development and growth. To account for developmental changes independent of the bubble sucking to breach transition, we subdivided each breathing mode into two age classes, expanding our analysis to include kinematic comparisons among four air-breathing stages: 'early bubble sucks', 'later bubble sucks', 'pre-metamorph breaches' and 'metamorph breaches'. These distinctions were made to accommodate substantial developmental changes that occur during tadpole ontogeny, particularly at early stages (e.g. rapid growth and development of the oral apparatus). Bubble sucks by tadpoles at NF stage 47–48 (the earliest observed breaths) were designated 'early', and those of all later stage tadpoles as 'late'. Metamorphs were identified as those tadpoles at NF stages ≥60. While tadpoles exhibiting 'later bubble sucks' and 'pre-metamorph breaches' overlapped somewhat in size, these groupings allowed us to distinguish among the effects of breathing mode, developmental stage and body size.

We used linear regression to model the effects of breathing stage and growth on the durations of the kinematic phases that make up an individual breath (suction, compression, lung filling and lung emptying). Our model terms included breathing stage as a four-level factor and the interaction between SVL and breathing stage, testing whether SVL has a significant effect within breathing stages. This method allowed us to account for growth within discrete developmental stages, while preventing body size from biasing the model at later developmental stages when tadpoles begin to decrease in SVL (Fig. 2). We adjusted SVL by fitting a linear model of SVL by breathing stage and then used the residuals as the SVL term in our main model of duration. Using the residuals avoids collinearity, i.e. the error associated with modeling the interaction between correlated predictor variables, in this case SVL and breathing stage. To avoid over-fitting, we performed a model selection test using AIC to compare three candidate models: (1) Duration~breathing stage; (2) Duration~breathing stage:resid (SVL~breathing stage); and (3) Duration~breathing stage+breathing stage:resid (SVL~ breathing stage).

For all kinematic phases in which breathing stage was found to be significant, we used the package 'emmeans' v.1.5.1 (https:// CRAN.R-project.org/package=emmeans) to examine the pair-wise comparisons with a Bonferroni correction to assess which breathing stages differed for each kinematic phase.
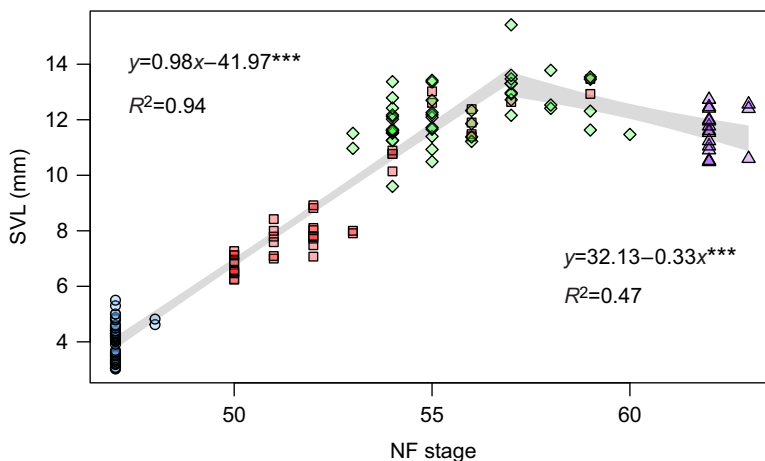

Fig. 2. Relationship of development and growth in *Xenopus laevis*. A breakpoints analysis reveals two distinct and independently significant linear relationships between growth (snout–vent length, SVL) and developmental stage (Nieuwkoop– Faber, NF stage). Growth peaks at NF stage 57, and then declines through metamorphic climax. Markers indicate breathing stages (blue circles: early bubble sucks; red squares: later bubble sucks; green diamonds: pre-metamorphic breaches; purple triangles: metamorphic breaches).
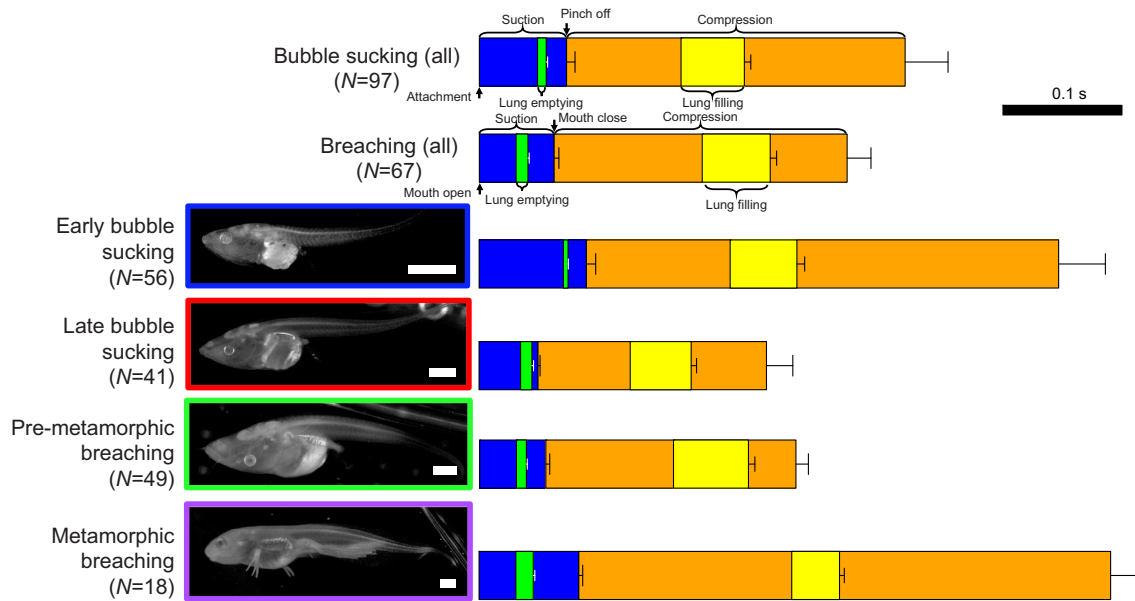
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Fig. 3. Average kinematic duration of air breathing across breathing modes and stages in *X. laevis*. Left: photos of each breathing stage. Scale bars: 2 mm. Right: bar diagrams of air breathing for total bubble sucking, total breaching, early bubble sucking, late bubble sucking, pre-metamorphic breaching and metamorphic breaching. Each bar is subdivided into explicit kinematic phases which are colored consistently. The length of each phase is the average duration (with standard error bars) as calculated from all analyzed videos.

RESULTS

Development of breathing behavior and associated lung morphology in *Xenopus laevis*

Following is a narrative description of the breathing mechanics and associated lung morphology for the four breathing stages identified above. Fig. 3 provides images of tadpoles and a standardized bar diagram summarizing air-breathing kinematics for each breathing stage. The bar diagrams represent the average duration of several important kinematic phases and events during an individual breathing bout. Tadpole air breathing typically begins with a 'suction phase', which is initiated either by attachment during bubble sucking or when the mouth opens after clearing the surface tension during breaching. During suction, the tadpole sucks air into the mouth by rapidly depressing the buccal floor to create negative pressure (Fig. 4A). The tadpole then empties the lungs (hereafter

referred to as 'lung emptying') and closes the mouth ('pinch off') (Fig. 4B,C). Once the mouth is closed, the tadpole increases intraoral pressure to compress the air, forcing it through the glottis and into the lungs (Fig. 4D,E). Once the lungs are full, any residual air left in the mouth is released as a bubble ('release'). The entire period from pinch off to release is the 'compression phase', but only the period during which air is actively moving into the lungs is considered 'lung filling'. Exact values of kinematic phase durations for different developmental periods can be found in Table 2.

Hatchlings (non-breathing; stages 45–46, <3.0 mm SVL)

Hatchling *X. laevis* tadpoles are minute and mostly non-sessile. They do not surface to breathe for several days after hatching, but after reaching ~2.7 mm SVL have begun lung development. The lungs at this stage are not inflated and have relatively thick, non-
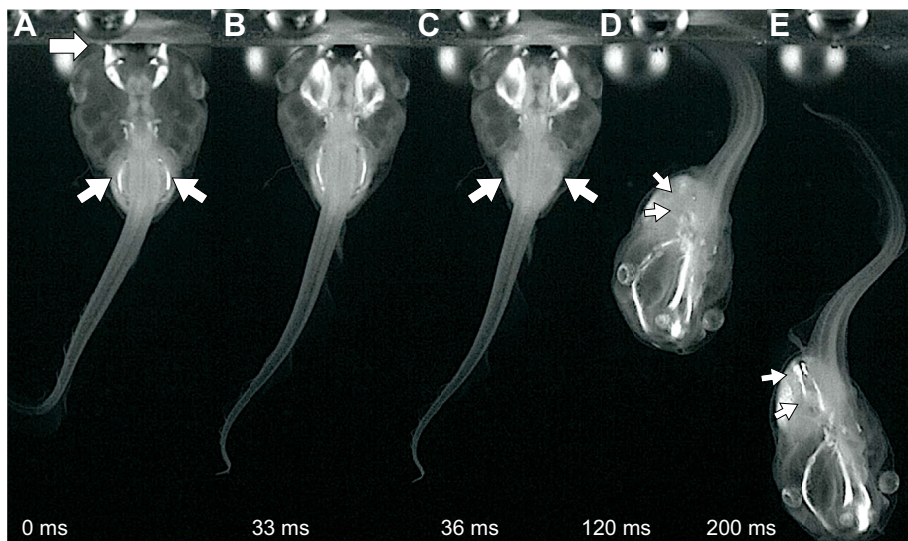

Fig. 4. Kinematic phases of bubble sucking in *X. laevis*. Images are taken from Movie 2. (A) Suction. Note the inflated lungs. (B,C) Lung emptying, which occurs during the suction phase. Note how the lungs empty while the air bubble is still connected to the surface. (D,E) The lungs fill during the compression phase. Modified from Schwenk and Phillips (2020).

Table 2. Mean (±s.d.) duration of kinematic phases for bubble sucks, breaches and their four subdivided breathing stages

Kinematic phase	Duration (s)					
	Bubble sucks (all) (N=97)	Breaches (all) (N=67)	EBS (N=56)	LBS (N=41)	PMBr (N=49)	MBr (N=18)
Suction	0.073±0.04	0.062±0.02	0.090±0.04	0.050±0.01	0.055±0.02	0.084±0.02
Compression	0.285±0.20	0.247±0.11	0.397±0.22	0.192±0.12	0.211±0.06	0.447±0.12
Lung emptying	0.007±0.01	0.010±3e−3	0.004±1e−3	0.010±8e−3	0.008±4e−3	0.015±7e−3
Lung filling	0.053±0.03	0.057±0.02	0.056±0.04	0.051±0.03	0.063±0.03	0.040±0.02
Suction I	0.049±0.04	0.031±0.01	0.071±0.05	0.035±0.01	0.031±0.01	0.031±0.01
Suction II	0.014±0.03	0.022±0.02	0.025±0.05	0.006±0.02	0.015±0.02	0.043±0.01
Total breath	0.352±0.22	0.312±0.12	0.496±0.25	0.240±0.06	0.264±0.06	0.545±0.11

EBS, early bubble sucks; LBS, late bubble sucks; PMBr, pre-metamorphic breaches; MBr, metamorph breaches. Suction I was defined as the period from attachment/mouth opening to the beginning of lung emptying. Suction II was defined as the period from the end of lung emptying to pinch off/mouth closure.

vascular walls (Fig. 5A). The lungs are large compared with the rest of the body and lie within the dorsal part of the coelom, extending posteriorly on either side of the central axis.

Early bubble sucking (stages 47–49, 3.0–5.5 mm SVL)

Tadpoles begin surfacing for air roughly 5 days after hatching, at approximately 3 mm SVL. They are unable to break the water's surface tension at this size and therefore breathe by bubble sucking, i.e. swimming up to the surface to attach and sucking air down into the mouth before turning quickly to the side to separate a bubble from the surface (Movie 1). Tadpoles create suction by depressing the buccal floor, which pulls the air–water interface down into the mouth, forming a bubble. At this early stage the bubble is small and round or ovoid. After pinch off, tadpoles elevate the buccal floor, compressing the bubble within the mouth. We often observed residual air in the lungs move anteriorly during the suction phase, but the lungs were not always emptied during these early breaths.

When lung emptying did occur, it took place during the suction phase, usually just before pinch off while the bubble was in the mouth and still connected to the water's surface. Following suction, all breaths include a longer compression phase in which the buccal floor is elevated, forcing air into the lungs if empty. After lung filling, the tadpole eventually releases excess air from the mouth as a bubble. The lungs at this point are inflated, but only partially, not yet forming the long, finger-like projections down the back typical of anuran tadpoles. There are no lung diverticula nor is there any discernible pulmonary vascularization in these tadpoles (Fig. 5B).

Late bubble sucking (stages 50–59, 5.5–13.5 mm SVL)

At ∼6 mm SVL, tadpoles continue to bubble suck, but several aspects of their air-breathing behavior differ from those of younger tadpoles (Movie 2). Following attachment, tadpoles suck air down from the surface and the lungs regularly empty just before, during, or occasionally just after the bubble is pinched off from the surface.
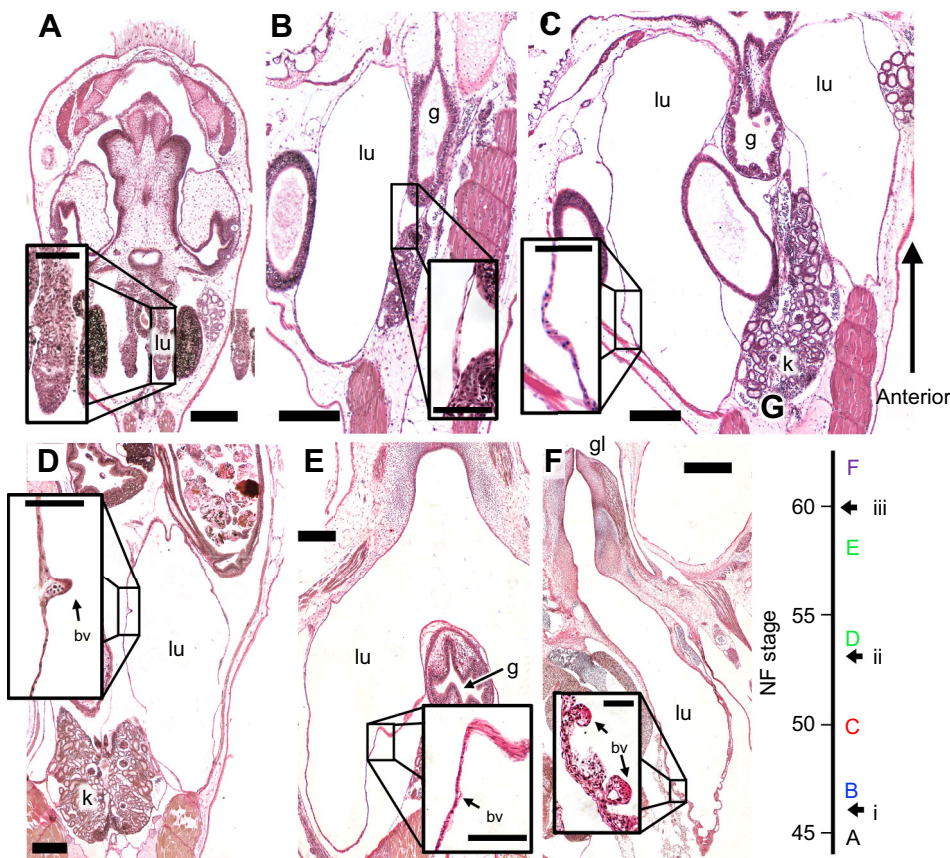

Fig. 5. Frontal sections through the lungs of *X. laevis* tadpoles at different stages. (A) Pre-breathing, NF stage 45 tadpole (2.6 mm SVL). Note the uninflated lung. (B) Early bubble sucking, NF stage 47 tadpole (5.0 mm SVL). The lungs are thin walled, with no discernible vasculature. (C) Late bubble sucking, NF stage 50 tadpole (7.5 mm SVL) with large, thin-walled lungs lacking obvious vasculature. (D) Pre-metamorphic breaching, NF stage 54 tadpole (12.5 mm SVL) with large, thin-walled lungs. There are some well-developed, large blood vessels, but not many. (E) Pre-metamorphic breaching, NF stage 58 tadpole (13.7 mm SVL) with few well-defined blood vessels. (F) Metamorphic breaching, NF stage 62 individual (12.2 mm SVL) with thick lung walls full of closely spaced, well-defined blood vessels. The scale on the right represents the stages of development of the tadpoles whose lungs are shown in A–F. Text colors represent breathing stage (black, pre-inflation; blue, early bubble sucks; red, later bubble sucks; green, pre-metamorphic breaches; purple, metamorphic breaths). i marks the onset of air breathing, ii marks the bubble suck to breach transition, and iii marks the onset of metamorphosis. lu, lung; g, gut; k, kidney; gl, glottis; bv, blood vessel. All sections were stained with Weigert iron hematoxylin and picro-ponceau (Presnell and Schreibman, 1997). Scale bars in A–F: 250 µm. Scale bars in insets: 100 µm (all insets but the first and last are at the same magnification).

The bubble is larger than in early bubble sucking and fills most of the buccal cavity, conforming to the internal relief of the mouth so that its shape is irregular. The bubble comprises several 'lobes' and clearly outlines the branchial baskets and roof of the mouth as it is pressurized. Compression refills the lungs and excess air is released. The lungs at this stage are long, extending far down the back. The lungs lack discernible vascularization but have begun to develop diverticula (Fig. 5C).

Pre-metamorphic breaching (stages 53–60, 9.6–15.4 mm SVL)
At ~10 mm SVL, tadpoles begin to occasionally breach the surface to breathe (Movie 3), and by 11.5 mm, they breach almost exclusively (Fig. 6B). Nevertheless, bubble sucking continues at a low frequency until the onset of metamorphic climax. Breaching lacks an attachment phase but is otherwise similar to bubble sucking, including both suction and compression phases in each breathing bout. During a breach, tadpoles swim rapidly upward to penetrate the surface tension, achieving a small period of 'air time' when the mouth is above the water, during which it opens, sucking in fresh, gaseous air. The lungs empty during the suction phase while the mouth is still open above the surface. As the tadpole falls back into the water, the mouth closes, and the breath is effectively pinched off. After this, breaching is indistinguishable from bubble sucking, with buccal air compressed to fill the lungs and the excess air released as a bubble. Breaching tadpoles empty and fill their lungs during each breath, virtually without exception. The lungs at this stage are very large and have exaggerated diverticula. The earliest occurring pulmonary blood vessels develop at this stage, but no pre-metamorphic tadpoles were found to have more than one or two capillaries visible within a single histological section (Fig. 5D).
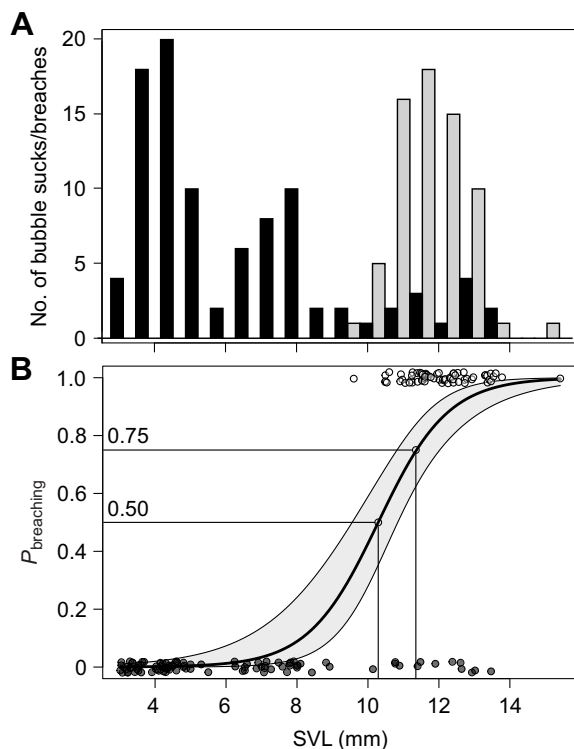

Fig. 6. The bubble suck to breach transition. (A) Histogram showing the number of recorded instances of bubble sucking (black) and breaching (gray). (B) The probability of breaching over ontogeny under a binomial GLM; 95% confidence intervals are indicated with gray shading. The probability of breaching exceeds 50% at ~10 mm SVL.

The lung walls of the largest breaching individuals stained a brighter pink than those of younger tadpoles, which might indicate the presence of more connective tissue than in younger individuals.

Metamorphic breaching (stages 61–64, 12.7–10.5 mm SVL)
Tadpoles continue to breach breathe throughout metamorphic climax. However, the method of breaking through the surface tension changes with the development of stronger legs and reduction of the tail (Movie 3). Metamorphic tadpoles swim up to the surface with symmetrical hindlimb strokes and then push rhythmically with both hindfeet against the surface multiple times, terminating with two rapid kicks to launch themselves up and through the surface while opening the mouth to draw in air. After the surface has been broken, metamorphic breaching is generally similar to pre-metamorphic breaching, although we did find some statistically significant differences in the duration of certain kinematic phases (see below). Compared with younger tadpoles, metamorphs empty their lungs relatively early during the suction phase, resulting in a longer period after the lungs have emptied during which the mouth remains open to the atmosphere prior to mouth closure. During metamorphic climax, the lungs undergo rapid and significant changes. The lungs of metamorphic tadpoles we examined (N=2) were thick walled and highly vascularized. In a single histological section through the frontal plane, we observed 20+ vessels in the wall of the lung. The vessels appear to form a net-like structure around the lungs (Fig. 5E).

Summary of lung development
The lung walls did not become significantly vascularized until approximately NF stage 61, during metamorphosis. No significant vascularization was observed in any pre-metamorphic individuals, regardless of breathing mode. The transition from avascular to vascular lungs happens very rapidly – one individual at NF stage 58 exhibited practically no vascularization, while a second at NF stage 62 had well-vascularized lungs (Fig. 5E,F). The lungs change shape throughout ontogeny, elongating, becoming septate and developing dorsal diverticula, which are maintained until metamorphosis, at which point they disappear. During metamorphosis, the lungs also come to lie more ventrally within the body cavity, ventral, rather than lateral, to the axial musculature along the midline of the body.

Ontogenetic changes in air breathing
Bubble sucking to breach transition
We first examined a histogram of body sizes for the two breathing modes over ontogeny (Fig. 6A), which confirmed that bubble sucking is replaced by breaching midway through ontogeny (as suggested by Schwenk and Phillips, 2020). Tadpoles first breathe exclusively by bubble sucking but eventually transition to breach breathing. The smallest tadpole observed breaching was 9.6 mm SVL. We found that breathing mode was highly correlated with both SVL and NF stage, and that tadpoles become more likely to breach than bubble suck at roughly 10.3 mm SVL (Fig. 6B), between NF stages 53 and 54. At approximately 11.4 mm SVL and between NF stages 55 and 56, tadpoles have a 75% likelihood of breaching when breathing. Bubble sucking continues at a low frequency until metamorphosis begins. We did not observe bubble-sucking behavior in any metamorphic individuals.

Bubble sucking versus breaching
When bubble sucking and breaching were compared by kinematic phase duration in a paired t-test, the mean duration for most kinematic phases differed significantly between the two breathing
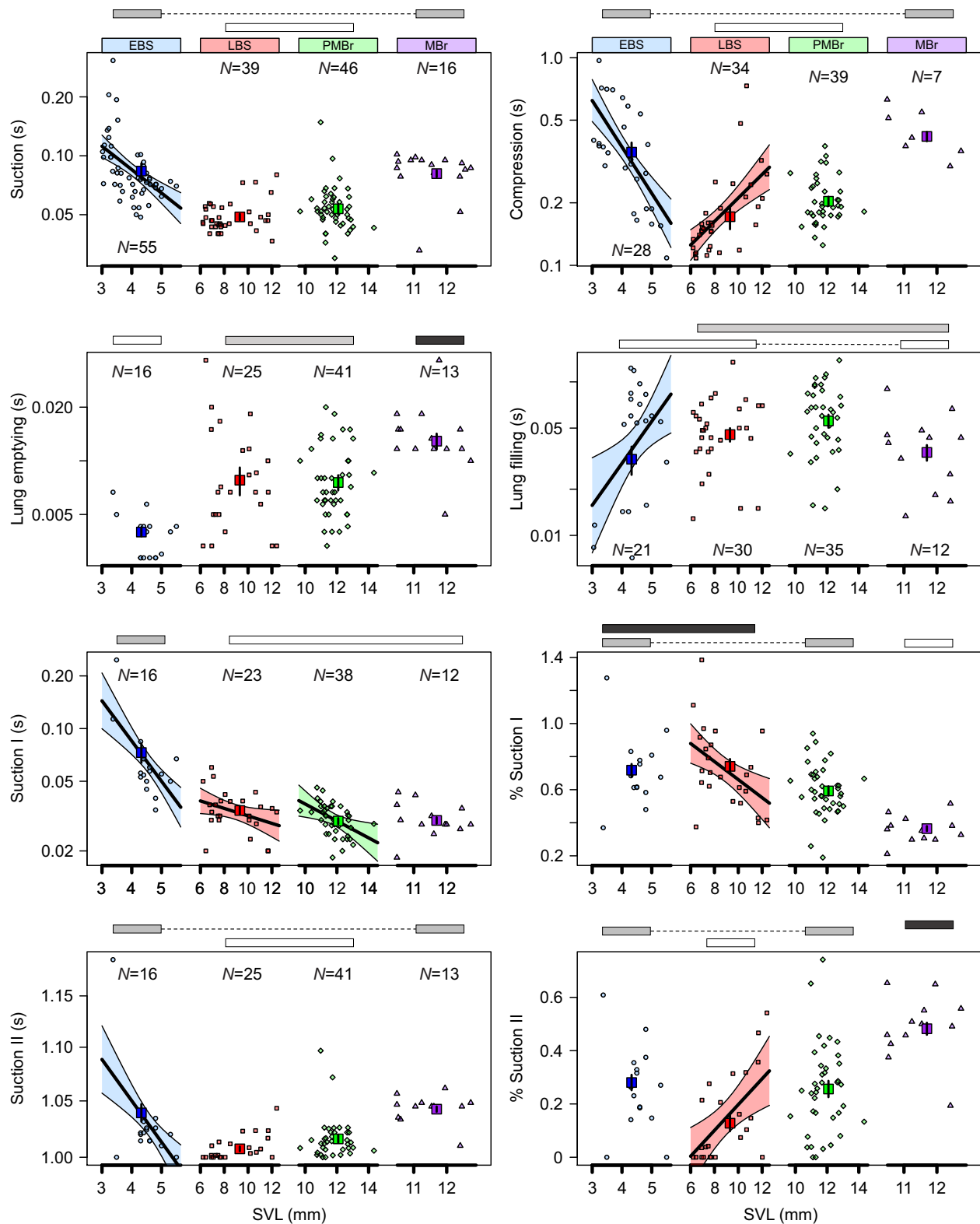
Fig. 7. Regressions of duration by breathing stage and size (SVL). Kinematic duration data are grouped first by breathing stage and then SVL within each breathing stage on the x-axis. EBS, early bubble sucks; LBS, late bubble sucks; PMBr, pre-metamorphic breaches; MBr, metamorphic breaches. The y-axis is on the log scale except for the percentage-based kinematics. Regression lines and 95% confidence intervals are only shown for breathing stages in which SVL had a significant effect on duration. Least square means are plotted with standard error as the large squares for each breathing stage. The bars above each plot represent groups of breathing stages that did not differ significantly in duration from one another in a *post hoc* test with a Bonferroni correction. Lighter bars denote groups with shorter average durations and darker bars those with longer average durations.

modes but only slightly (all differences were less than 0.04 s; Table S1). When we excluded early bubble sucks and metamorphic breaths, only comparing later bubble sucks to pre-metamorphic breaches, we found only slight, non-significant differences in both positive and negative directions between the mean durations for all kinematic phases (Table S2), suggesting there are no sudden changes in duration at the bubble suck to breach transition.

Suction and compression duration
The developmental patterns of suction and compression are similar and can be described together. In both cases, breathing stage and the interaction of breathing stage and adjusted SVL (see Materials and Methods – henceforth referred to simply as SVL) were significant predictors of duration for suction and compression phases. The four breathing stages split into two similar groups by duration: early bubble sucks and metamorphic breaches had longer durations of suction and compression than later bubble sucks and pre-metamorphic breaches. Within each pair of breathing stages, mean duration did not differ, while between pairs, duration differed significantly (Fig. 7, Table 3; Table S2).

The durations of suction and compression also changed similarly with growth (SVL) during the early bubble-sucking stage. SVL had a significant, negative interaction term (slope) for both suction and compression duration. The only other significant SVL interaction for suction or compression was late bubble sucks, for which SVL was significant with a positive slope (Fig. 7, Table 3; Table S2).

Lung-emptying duration
The lung-emptying phase was the only phase in which SVL had no significant effect on duration. Breathing stage alone explained roughly a third of the variation in duration of the lung-emptying phase across ontogeny (adjusted $R^2=0.3471$). Lung-emptying duration was shortest in early bubble sucks, did not differ between later bubble sucks and pre-metamorphic breaches, and was longest in metamorphic breaches. Duration differed significantly between all pairings except later bubble sucks and pre-metamorphic breaches (Fig. 7, Table 3; Table S2).

Lung-filling duration
The lung-filling phase (Fig. 7, Table 3) had the least ontogenetic change in duration of all the kinematic phases that we observed. The only breathing stages that differed significantly in lung-filling duration were early bubble sucks and pre-metamorphic breaches, and only early bubble sucks had a significant (and positive) interaction with SVL on duration (Fig. 7, Table 3; Table S2).

Timing of lung-emptying during suction (suction I and II duration)
Lung emptying virtually always began during the suction phase. The duration of suction before lung emptying began (suction I) was longest in early bubble sucks compared with all other breathing stages. This duration only differed significantly for pairwise comparisons that included early bubble sucks. Additionally, the interactions between SVL and the first, second and third breathing stages were all significant and negative for suction I. When converted to the relative timing of lung-emptying by dividing suction I by total suction duration, the relative duration of suction before lung-emptying was shortest in metamorphic breaches, which differs from all other breathing stages. Early bubble sucks and pre-metamorphic breaches also differed significantly in the relative duration of suction before lung emptying. The interaction between SVL and breathing stage for the relative duration of suction I was only significant (and negative) for later bubble sucks (Fig. 7, Table 3; Table S2).

After the lungs emptied, tadpoles either continued to hold their mouth open, which we counted as part of the suction phase, or closed their mouth just before or as the lungs fully emptied. The mean duration of suction after lung emptying (suction II) was greatest in early bubble sucks and metamorphic breaches (which did not differ significantly) and lowest in late bubble sucks and pre-metamorphic breaches, which did not differ significantly. Only early bubble sucks had a significant (negative) interaction between SVL and breathing stage for suction II. When converted to relative timing by dividing suction II by total suction duration, the relative duration of the period following lung emptying was significantly longer in metamorphic breaches than in any other breathing stage. Early bubble sucks versus late bubble sucks, as well as late bubble sucks versus breaching also differed significantly in the relative duration of suction after lung emptying. The interaction between SVL and breathing stage was only significant (and positive) for later bubble sucks. Across our analyses, later bubble sucks and pre-metamorphic breaches only differed significantly in the relative duration of suction I and suction II (Fig. 7, Table 3; Table S2).

DISCUSSION
Ontogenetic changes in breathing mechanics
After accounting for the effects of growth, the only functionally significant difference between bubble sucking and breaching we could discern is the method of air acquisition. *Xenopus laevis* tadpoles initially breathe air by bubble sucking, but after significant growth and development, they transition to breaching. Comparisons between these breathing modes are therefore confounded by other developmental changes. While we were able to detect statistically

Table 3. Effect sizes and *P* values of model predictors for different kinematic phases

Kinematic phase	Breathing stage		Breathing stage and SVL interaction	
	Sum of squares	*P*-value	Sum of squares	*P*-value
Suction	1177.44	<2.2e−16***	2.06	1.342e−05***
Compression	237.76	<2.2e−16***	6.651	3.7e−10***
Lung emptying	NA	NA	NA	NA
Lung filling	878.08	<2.2e−16***	3.39	0.076
Suction I	984.34	<2.2e−16***	2.14	5.962e−06***
% Suction I	32.914	<2.2e−16***	0.353	0.03081*
Suction II	0.0502	4.04e−16***	0.0076	0.00129**
% Suction II	6.2501	<2.2e−16***	0.2728	0.02729*

Lung emptying was the only kinematic phase for which the SVL×breathing stage interaction was not supported in the final model. Suction I was defined as the period from attachment/mouth opening to the beginning of lung emptying. Suction II was defined as the period from the end of lung emptying to pinch off/mouth closure.

significant differences between bubble sucks and breaches for the duration of most kinematic phases, the magnitude of these effects makes it unlikely these differences have functional implications for air breathing (Table S1). Additionally, we did not find any significant differences in raw duration between later bubble sucks and pre-metamorphic breaches, which are the most analogous subsets of the two breathing modes (Fig. 7; Table S2).

Of the patterns we observed using the four breathing-stage model (Fig. 5), we believe the following four results to be most important in understanding the air-breathing biology of *X. laevis*, (1) We found that the very earliest breaths (the very first early bubble sucks) had the longest average duration and the longest duration of the suction and compression phases. As tadpoles grow and mature, however, these durations decrease sharply over the first breathing stage. This is shown by the significant, negative effects of SVL on duration for suction and compression during breathing stage I. This result may be a consequence of early growth and/or muscle maturation, which would allow tadpoles to exert more force on the surface tension to capture a bubble more quickly and/or compress it more forcefully. (2) Later bubble sucks and pre-metamorphic breaches did not differ in duration for any kinematic phases. Notably, it was neither faster to capture gaseous air than a bubble from the surface nor faster to compress the air into the lungs for either breathing mode. (3) Total breath duration of metamorphic individuals was most similar to that of early bubble suckers (although shorter in duration than breaths of the youngest early bubble suckers) and significantly longer than the duration of the intermediate breathing stages. The increase in breath duration between pre-metamorphic and metamorphic breaches is not correlated with growth within a stage, but instead appears to be a discrete kinematic change related to the onset of metamorphosis. During metamorphosis, both the mechanics of breathing and the internal morphology are rapidly changing, so it is not surprising that the duration of certain kinematic phases also changes. (4) Finally, the kinematics of lung ventilation also differ in metamorphs compared with all other breathing stages. In metamorphs, the relative timing of lung emptying shifts to earlier in the suction phase compared with previous stages. Metamorphs, on average, spend more time with their mouths open to the air following lung emptying than all other breathing stages. This extra time may allow expired air to be more diluted by atmospheric air prior to inspiration. With less time for mixing and dilution, breaths of earlier stages potentially have a lower capacity for gas exchange. Although early bubble sucks do not differ from those of metamorphs in total duration of the suction/inspiration phase, the proportion of time after lung emptying is significantly longer in metamorphs.

Is the onset of breaching correlated with a physiological transition in respiratory mode?

Hastings and Burggren (1995) showed that *X. laevis* tadpoles transition from oxygen conformers to oxygen regulators at NF stage 53–57. Our analysis suggests that *X. laevis* tadpoles transition from bubble sucking to breaching at NF stages 53–54, which aligns well with the physiological transition to oxygen regulation. This circumstantial evidence suggests that breaching might allow for greater pulmonary gas exchange capacity than bubble sucking. Alternatively, it is possible that both transitions are related to a third developmental phenomenon, or that the correlation is coincidental.

The best proxy of gas exchange performance we have available is lung morphology and development. We did not find evidence for a shift in lung morphology correlated with the onset of breaching. Breaching tadpoles are, overall, larger than bubble-sucking individuals and larger tadpoles have larger, more septate lungs

(also noted by Hastings and Burggren, 1995). However, the increase in lung size and septation occurred throughout growth without any sharp or notable change at the transition to breach breathing. Furthermore, there was no increase in lung vascularization associated with breaching. While it is possible that an increase in lung size and septation achieved a functionally significant threshold in breaching tadpoles, without an increase in vascularization it is unclear how a significant improvement in gas exchange could have occurred.

Despite finding that the mechanical bubble suck to breach transition aligns with the physiological transition found by Hastings and Burggren (1995), we found no evidence, either kinematic or morphological, to support the same physiological change in the tadpoles observed in this study. The lungs of breaching tadpoles were not obviously better equipped for gas exchange, and the kinematics of air breathing did not differ more than trivially between bubble sucking and breaching at comparable stages of development. A possible explanation for this apparent discrepancy is that Hastings and Burggren (1995) subjected tadpoles to acute hypoxia to test their ability to regulate blood oxygen levels, while we maintained tadpoles in normoxic conditions. In our study, we found a significant jump in both lung vascularization and potential kinematic gas exchange proficiency only at metamorphosis. Conceivably, acute hypoxia might have induced a similar, plastic response in earlier stage tadpoles at the bubble suck to breach transition point, particularly if only tadpoles large enough to breach are developmentally competent to respond plastically or if breaching provides a greater potential for gas exchange. Notably, the rapid morphological and kinematic changes we observed at metamorphosis correspond to the loss of respiratory buccopharyngeal surfaces, including the gills, for oxygen uptake, which would presumably also induce oxygen stress. As such, oxygen stress in developmentally competent tadpoles might induce the phenotypic changes necessary for a transition to oxygen regulation, either at metamorphosis under normoxic conditions or earlier at the bubble suck to breach transition under hypoxic conditions.

Evolution of the bubble suck to breach transition

Xenopus laevis tadpoles, like many other anurans, begin air breathing at very small body sizes (Ultsch et al., 1999; Schwenk and Phillips, 2020). At these small sizes, breaching is made physically impossible by surface tension, which prevents tadpoles from breaking through the surface to breathe (Schwenk and Phillips, 2020). By metamorphosis, most tadpoles are large enough to overcome the resistance of surface tension and breaching becomes possible. Some anurans, such as *X. laevis* and *Rana clamitans*, make a fairly clean transition to breaching, presumably as soon as possible (Schwenk and Phillips, 2020). At least one species, however (*H. versicolor*), never transitions to breaching, instead shifting from typical bubble sucking to a derived form of bubble sucking that includes a second suction event immediately following the first ('double bubble sucking'; Phillips et al., 2020). In other words, a transition to breach breathing is neither necessary nor inevitable. Why, then, do they breach? Is the transition to breach breathing adaptive? It may be that breaching has advantages for gas exchange, but that difference is only meaningful if tadpoles are using their lungs for a significant portion of their total gas exchange needs. If the oxygen content of the water is high enough, then cutaneous and branchial respiration are probably more than sufficient.

If breaching has a greater potential for gas exchange than bubble sucking, this may only be functionally relevant at critically low levels of environmental oxygen. Future studies should examine the potential role of plasticity in both breathing mechanics and lung

development mediated by oxygen stress. It may be that the onset of breaching behavior marks the earliest point at which respiratory air breathing is possible in tadpoles such as *X. laevis*. All things being equal, typical tadpoles should breach at approximately the same body size (~10 mm SVL in *X. laevis*), the size at which surface tension no longer prohibits breaching. Tadpoles that do not breach, such as *H. versicolor*, nevertheless transition to an alternative breathing mode (double bubble sucking), which clearly increases potential pulmonary gas exchange capacity (Phillips et al., 2020). By shifting to a form of bubble sucking rather than breach breathing, *H. versicolor* tadpoles eliminate the minimum body size required by surface tension to breach, and instead transition to a breathing mode with advantages for gas exchange at only 6 mm SVL (significantly smaller than the transition point for breaching).

All examined tadpoles undergo some developmental transition in breathing mechanics, whether to breaching or to another form of bubble sucking (Schwenk and Phillips, 2020; Phillips et al., 2020). In breaching tadpoles, the transition point from bubble sucking is fixed by surface tension. Many arboreal frogs, particularly those that breed in anoxic micro-bodies of water, are obligate air breathers as tadpoles (Noble, 1929; Lannoo, 1987). These species, which include *Hoplophryne* and some members of *Osteopilus*, among others (Lehtinen et al., 2004), often share a common set of traits, such as a narrow snout, elongated body and strong tail musculature. These attributes have previously been identified as locomotory adaptations associated with the high viscosity of the phytotelma caused by egg jelly and detritus (Noble, 1929; Lehtinen et al., 2004), but they might also permit earlier breaching.

Xenopus in the context of vertebrate ventilation pumps

Anamniote vertebrates, including anurans, ventilate the lungs with a buccal pump, which uses positive pressure created by compression of the buccopharyngeal chamber to fill the air-breathing organs (Brainerd, 1994, 1999; Liem, 1988). However, there is substantial diversity in the form of buccal pumps among vertebrates, which differ among phylogenetic lineages and ecological groups. Some groups use two or more expansion and compression cycles during a breath to separate inspired and expired airstreams, preventing the mixing of fully oxygenated air with the de-oxygenated, residual air expelled from the lungs, a respiratory mode Brainerd (1994) called 'four-stroke breathing'. Organisms that breathe using a four-stroke pump include many actinopterygian fishes (Liem, 1988), some aquatic salamanders (*Amphiuma*: Toews and McRae, 1974; *Cryptobranchus*: E. L. Brainerd, personal communication), *H. versicolor* tadpoles (Phillips et al., 2020) and the adult pipid frogs *X. laevis* and *Pipa carvalhoi* (Boutilier, 1984; Brett and Shelton, 1979; Fonseca et al., 2012). Most anamniote vertebrates, including lungfish and all other previously studied amphibians (including other adult frogs), employ only a single expansion and compression cycle, known as 'two-stroke' breathing, presumed to be the ancestral condition for Sarcopterygia (Brainerd, 1994).

In *X. laevis* tadpoles, we observed only two-stroke breathing typical of sarcopterygian fishes and other non-hylid tadpoles (see Phillips et al., 2020, for a summary). However, Brett and Shelton (1979) and Boutilier (1984) found that the aquatic adults of *X. laevis* separate inspiration from expiration, leading Brainerd (1999) to classify *X. laevis* as a four-stroke air breather. Larval *X. laevis* therefore transition from ancestral two-stroke breathing to derived, four-stroke breathing at some point after metamorphosis. This transition makes functional sense because the presence of gills and high cutaneous surface area to volume ratio in tadpoles makes the separation of airstreams provided by four-stroke breathing

redundant, whereas in larger, gill-less adults, with a lower cutaneous surface area to volume ratio, efficient pulmonary gas exchange is probably more important. There could be significant selection for increased pulmonary capacity in the aquatic adults, which cannot always rely on well-oxygenated water for cutaneous respiration. Most of the few documented examples of four-stroke breathing among sarcopterygians are fully aquatic salamanders that lack robust gills, such as *Cryptobranchus* and *Amphiuma*, which apparently take advantage of a second two-stroke cycle to decrease the mixing of air streams and thus increase the efficiency of pulmonary gas exchange (Brainerd, 1999).

There are other mechanisms besides four-stroke breathing that increase respiratory efficiency, also found in large, aquatic salamanders with reduced gills, such as *Siren* (Brainerd and Monroy, 1998). While the breathing pattern of *Siren* is undoubtably two-stroke, it has moved the timing of lung emptying to earlier in the inspiration phase (analogous to our 'suction' phase), after which it inspires additional fresh air, which is then compressed to fill the lungs with minimal mixing. We found that at metamorphosis, lung emptying in *X. laevis* similarly shifts to a relatively earlier point in the suction phase, though not as extremely as in *Siren*. This could be a plastic response to oxygen stress, as discussed above, or it could be an intermediate phenotype between larva and adult, and two- and four-stroke air breathing. Regardless, we hypothesize that *X. laevis* undergoes a three-stage ontogenetic transition from two-stroke breathing larvae to four-stroke breathing adults via a shift in the timing of lung emptying relative to suction/inspiration. Lung emptying in pre-metamorphic tadpoles occurs very late in the suction/inspiration phase, then shifts to midway through the suction/inspiration phase in metamorphs, and then lung emptying eventually fully precedes inspiration in adults with the addition of a second expansion/compression cycle, resulting in four-stroke breathing.

There are very few known developmental transitions from two- to four-stroke breathing in the natural world (Phillips et al., 2020). The transition we describe here in *X. laevis* is particularly noteworthy because it is relatively gradual and may provide insight into how species transition evolutionarily between these two breathing modes. In the case of *X. laevis*, the gradual progression of the trait may be functionally adaptive throughout development. The change in timing of lung emptying might not only potentially serve as a transition to an adaptive adult state but could also increase the efficiency of air breathing in metamorphs, by increasing the time spent with the buccal bubble open to the atmosphere, mixing with fresh air and increasing its oxygen content before compression into the lungs. It is also possible that ancestral pipid adults retained the metamorphic breathing mechanism before evolving the derived, four-stroke breathing mode evident today, although this remains purely speculative. Although we now have data for breathing in both larval and adult *X. laevis*, direct comparisons are difficult because previous studies of adult pipids employed a pneumotachographic approach (i.e. measuring the flow of air into and out of the lungs) on large individuals (Boutilier, 1984; Brett and Shelton, 1979; Fonseca et al., 2012). We believe that direct visualization of breathing ontogeny with x-ray videography will allow us to characterize more accurately the transition from larval to adult breathing in *X. laevis* and other anurans.

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Table S1. Results of Welsh's two-sample T test.

Kinematic phase	Breathing Mode	T test estimate (s)	Magnitude of effect (s)	P value
Suction duration	Bubble-sucking	0.073	0.010	0.034 *
	Breaching	0.063		
Compression duration	Bubble-sucking	0.285	0.038	0.20
	Breaching	0.246		
Lung Emptying duration	Bubble-sucking	0.007	0.003	0.04
	Breaching	0.010		
Lung Filling duration	Bubble-sucking	0.053	0.004	0.51
	Breaching	0.057		
Suction I duration[1]	Bubble-sucking	0.049	0.018	3.2e-3 **
	Breaching	0.031		
Suction II duration[2]	Bubble-sucking	0.014	0.009	0.15
	Breaching	0.023		
Total breath duration	Bubble-sucking	0.352	0.040	0.24
	Breaching	0.312		

[1] Suction I is the first part of the suction phase, from attachment/mouth open to the start of lung emptying.
[2] Suction II is the final part of the suction phase, from the end of lung emptying to pinch-off/mouth closure.

Table S2. Model outputs from the linear regression of phase duration (s) by the four-level factor breathing stage and the interaction between SVL and breathing stage. SVL has been adjusted by first regressing SVL by breathing stage, and then using the residuals of that linear model as the "SVL" vector in the models presented here, so as to avoid collinearity. EBS = Early bubble-sucking, LBS = Later bubble-sucking, PMB = Pre-metamorphic breaching, and MB = Metamorphic breaching. SE = standard error.

Kinematic phase	Breathing stage	Breathing Stage estimate ± SE		Breathing stage and SVL estimate ± SE	Breathing stage and SVL p value	Breathing Stage pairwise comparisons P value	
(dependent)		log(e) scale	linear scale (s)			(with Bonferonni correction for 6 tests)	
Suction duration	EBS:	-2.483 ± 0.036	0.083	-0.294 ± 0.057	6.93e-7 ***	EBS - LBS: <.0001 ***	EBS - PMB: <.0001 ***
	LBS:	-3.021 ± 0.042	0.049	0.022 ± 0.019	0.245	EBS - MB: 1.000	LBS - PMB: .564
	PMB:	-2.926 ± 0.038	0.054	-0.051 ± 0.037	0.174	LBS - MB: <.0001 ***	PMB - MB: <.0001 ***
	MB:	-2.512 ± 0.065	0.081	-0.044 ± 0.083	0.592		
Compression duration	EBS:	-1.025 ± 0.089	0.359	-0.548 ± 0.089	1.38e-8 ***	EBS - LBS: <.0001 ***	EBS - PMB: <.0001 ***
	LBS:	-1.769 ± 0.025	0.171	0.119 ± 0.025	4.35e-6 ***	EBS - MB: 1.000	LBS - PMB: .153
	PMB:	-1.592 ± 0.050	0.203	-0.002 ± 0.050	0.976	LBS - MB: <.0001 ***	PMB - MB: <.0001 ***
	MB:	-0.864 ± 0.151	0.422	-0.227 ± 0.151	0.137		
Lung Emptying duration[1]	EBS:	-5.580 ± 0.120	3.77E-03	NA	NA	EBS - LBS: .0001 ***	EBS - PMB: <.0001 ***
	LBS:	-4.874 ± 0.098	7.65E-03	NA	NA	EBS - MB: <.0001 ***	LBS - PMB: 1.000
	PMB:	-4.887 ± 0.075	7.54E-03	NA	NA	LBS - MB: .0048 **	PMB - MB: <.0013 **
	MB:	-4.313 ± 0.128	0.013	NA	NA		
Lung Filling duration	EBS:	-3.482 ± 0.168	0.031	0.672 ± 0.237	0.00574 **	EBS - LBS: .402	EBS - PMB: .025 *
	LBS:	-3.094 ± 0.114	0.045	0.026 ± 0.053	0.629	EBS - MB: 1.000	LBS - PMB: 1.000
	PMB:	-2.889 ± 0.106	0.056	0.031 ± 0.115	0.786	LBS - MB: 1.000	PMB - MB: .154
	MB:	-3.356 ± 0.179	0.035	-0.148 ± 0.222	0.508		
Suction I duration[2]	EBS:	-2.502 ± 0.084	0.082	-0.564 ± 0.117	5.57e-6 ***	EBS - LBS: <.0001 ***	EBS - PMB: <.0001 ***
	LBS:	-3.371 ± 0.051	0.034	-0.045 ± 0.022	0.0410 *	EBS - MB: <.0001 ***	LBS - PMB: .187
	PMB:	-3.500 ± 0.039	0.030	-0.095 ± 0.036	0.0104 *	LBS - MB: .8585	PMB - MB: 1.000

	MB:	-3.503 ± 0.069	0.030	-0.038 ± 0.089	0.6716		
	EBS:	0.046 ± 6.69e-3	0.048[4]	-0.038 ± 9.26e-3	8.99e-5 ***	EBS - LBS: .0001 ***	EBS - PMB: .0045 **
Suction II duration[3,4]	LBS:	6.68e-3 ± 4.23e-3	0.007[4]	-2.85e-3 ± 1.78e-3	0.113	EBS - MB: 1.000	LBS - PMB: .641
	PMB:	0.016 ± 3.19e-3	0.016[4]	-9.81e-4 ± 2.91e-3	0.737	LBS - MB: .0001 ***	PMB - MB: .0025 **
	MB:	0.042 ± 5.76e-3	0.043[4]	-2.10e-3 ± 7.20e-3	0.772		
		percent of suction					
Suction I relative duration (% of suction)[5]	EBS:	71.9 ± 6.03%		-4.58e-3 ± 0.083	0.956	EBS - LBS: 1.000	EBS - PMB: .211
	LBS:	75.0 ± 3.69%		-0.050 ± 0.016	0.002 **	EBS - MB: .0001 ***	LBS - PMB: .012 *
	PMB:	59.7 ± 2.84%		-0.026 ± 0.026	0.321	LBS - MB: <.0001 ***	PMB - MB: .0026 **
	MB:	36.2 ± 5.18%		0.014 ± 0.065	0.832		
Suction II relative duration (% of suction)[5]	EBS:	29.6 ± 5.22%		-0.075 ± 0.072	0.304	EBS - LBS: .036 *	EBS - PMB: 1.000
	LBS:	11.8 ± 3.30%		0.044 ± 0.014	0.002 **	EBS - MB: .015 *	LBS - PMB: .015 *
	PMB:	25.36 ± 2.49%		0.012 ± 0.023	0.605	LBS - MB: <.0001 ***	PMB - MB: .0005 ***
	MB:	48.40 ± 4.49%		-8.86e-3 ± 0.056	0.875		

[1] Lung emptying was the only phase for which the SVL:breathing stage interaction was not significant and so not included in the final model.

[2] Suction I is the first part of the suction phase, from attachment/mouth open to the start of lung emptying.

[3] Suction II is the final part of the suction phase, from the end of lung emptying to pinch-off/mouth closure.

[4] Because some values in this dataset were equal to or less than zero, data was scaled up by adding 1, the linear values indicated have been rescaled, while the outputs on the log scale have not.
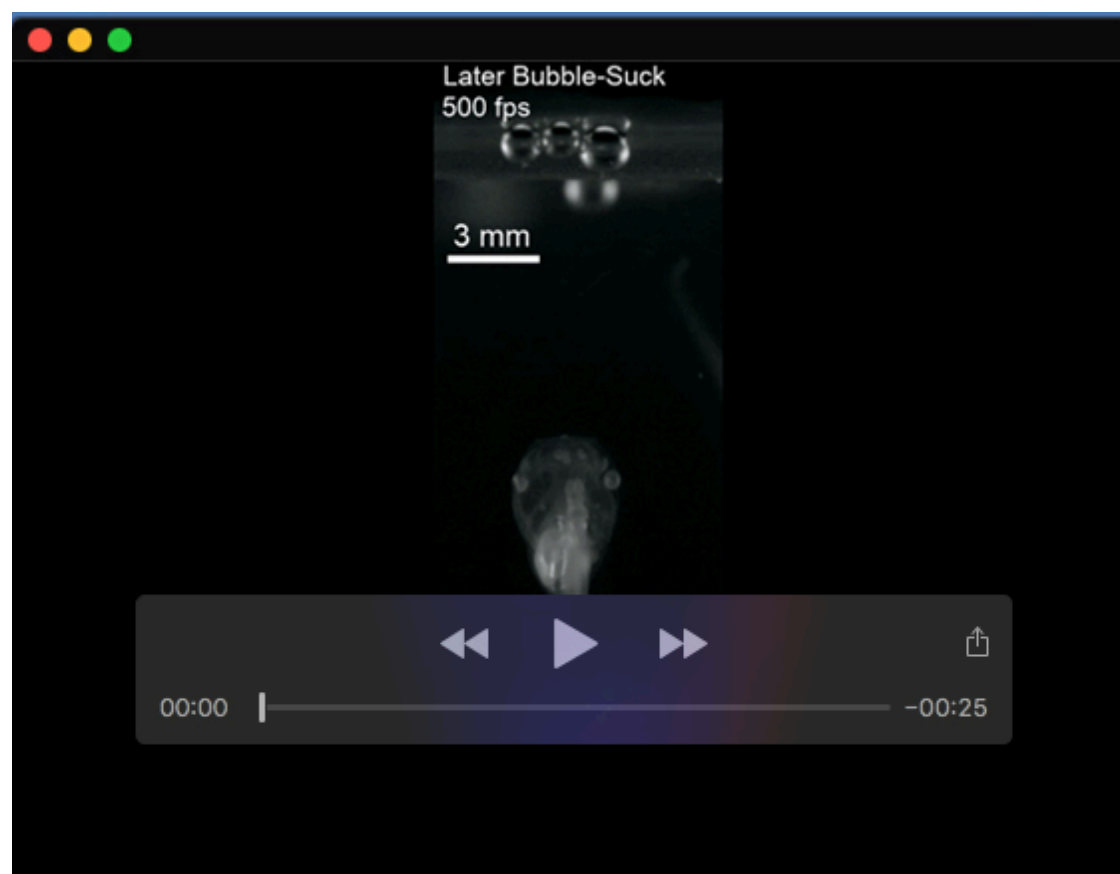
[5] These values have been rescaled by dividing either suction I or II by the total duration of suction, and were not log-transformed for analysis.
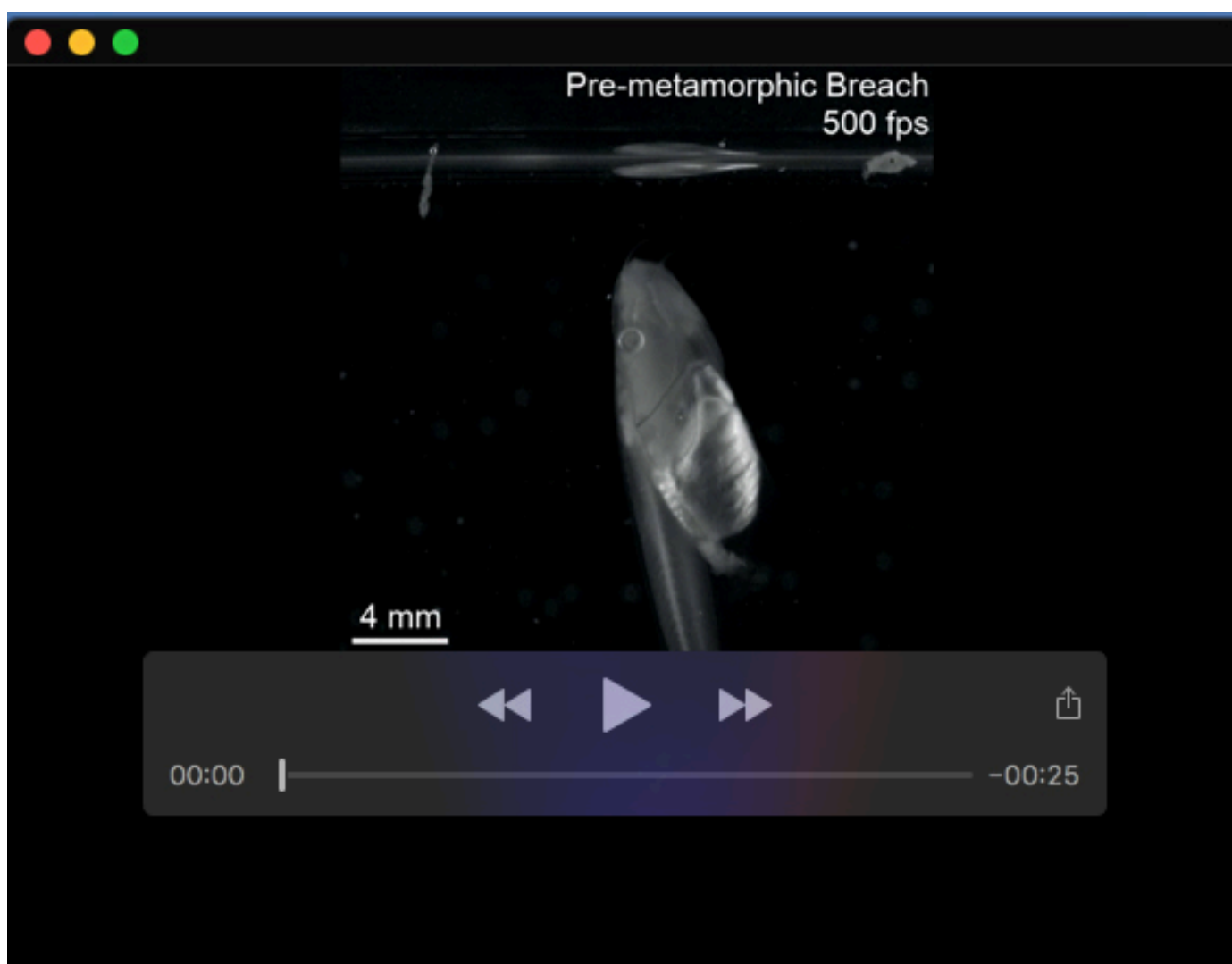
Table S3.

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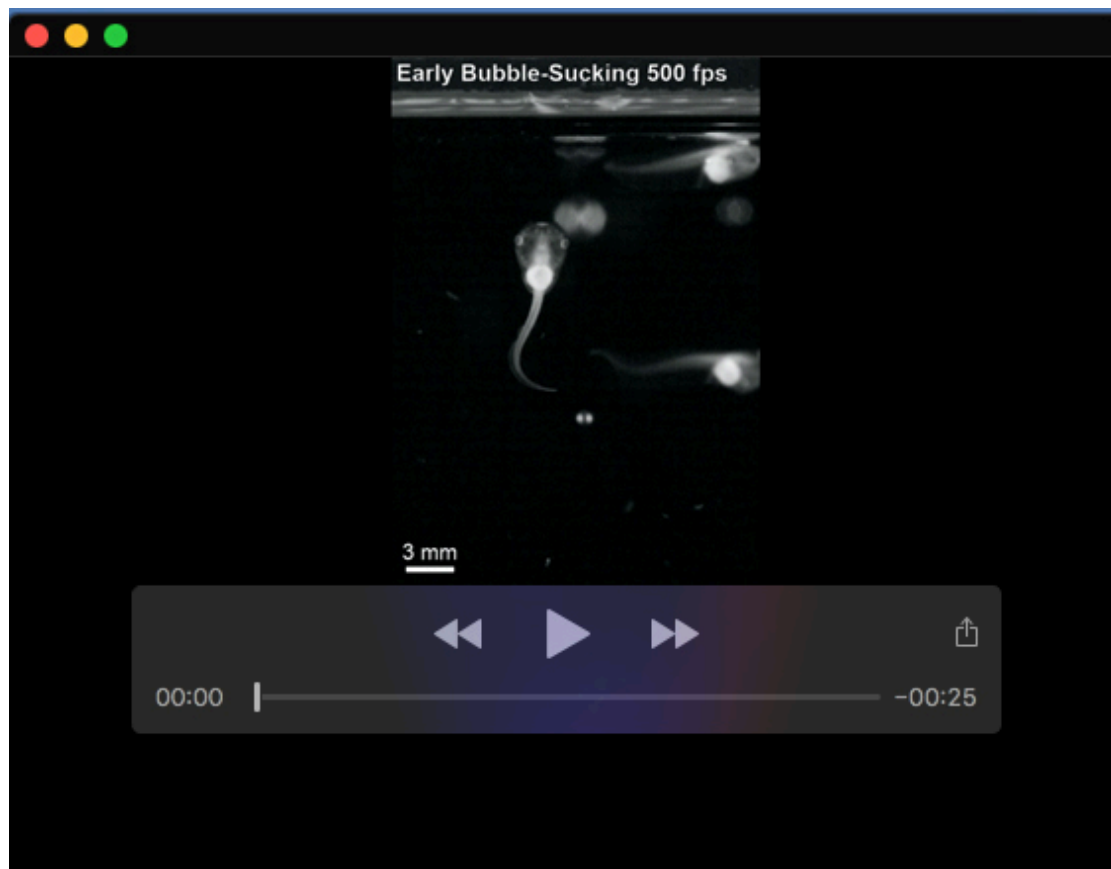
Movie 1. Highspeed video of an early bubble-sucking *Xenopus laevis* tadpole breathing by bubble-sucking.

Movie 2. Highspeed video of a later bubble-sucking *Xenopus laevis* tadpole breathing by bubble-sucking.

Movie 3. Highspeed video of a pre-metamorphic breaching *Xenopus laevis* tadpole breaching to breathe followed by a metamorphic *Xenopus laevis* individual breaching to breathe.