Report

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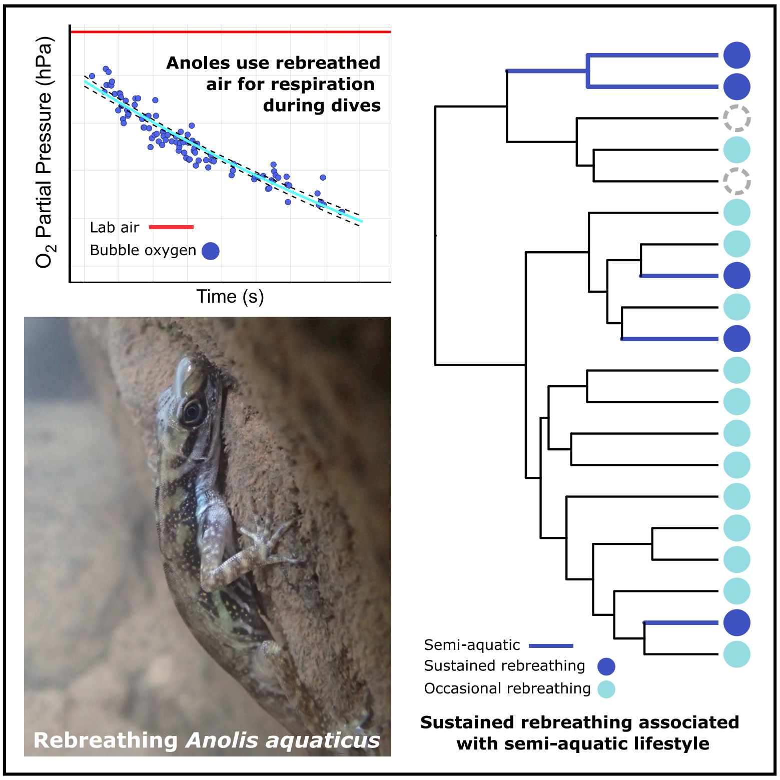
Repeated evolution of underwater rebreathing in diving *Anolis* lizards

Graphical abstract



Authors

Christopher K. Boccia, Lindsey Swierk, Fernando P. Ayala-Varela, ..., Andrés García, Richard E. Glor, D. Luke Mahler

Correspondence

christopher.boccia@mail.utoronto.ca (C.K.B.),
luke.mahler@utoronto.ca (D.L.M.)

In brief

Boccia et al. show that *Anolis* lizards can respire underwater by “rebreathing” exhaled air that is trapped between their skin and surrounding water. Many anole species occasionally rebreathe, but aquatic habitat specialists do so regularly. Phylogenetic analyses of rebreathing behavior suggest repeated adaptive convergence in diving anoles.

Highlights

- *Anolis* lizards can respire underwater by “rebreathing” exhaled air
- Many anole species occasionally rebreathe, but aquatic specialists do so routinely
- Phylogenetic analyses suggest regular rebreathing is adaptive in diving anoles

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Current Biology

CellPress

Report

Repeated evolution of underwater rebreathing in diving *Anolis* lizards

Christopher K. Boccia,[1,13,*] Lindsey Swierk,[2,3] Fernando P. Ayala-Varela,[4] James Boccia,[1] Isabela L. Borges,[5,6] Camilo Andres Estupiñán,[7] Alexandra M. Martin,[2] Ramón E. Martínez-Grimaldo,[8] Sebastian Ovalle,[7] Shreeram Senthivasan,[9] Ken S. Toyama,[1] María del Rosario Castañeda,[7] Andrés García,[10] Richard E. Glor,[11] and D. Luke Mahler[1,12,*]

[1]Department of Ecology and Evolutionary Biology, University of Toronto, ON M5S 3B2, Canada
[2]Department of Biological Sciences, Binghamton University, State University of New York, New York, NY 13902, USA
[3]Environmental Studies Program, Binghamton University, State University of New York, New York, NY 13902, USA
[4]Museo de Zoología, Escuela de Biología, Pontificia Universidad Católica del Ecuador, 12 de Octubre y Roca, Quito, Ecuador
[5]Department of Integrative Biology, College of Natural Sciences, Michigan State University, East Lansing, MI 48824, USA
[6]W.K. Kellogg Biological Station, Michigan State University, 3700 E. Gull Lake Drive, Hickory Corners, MI 49060, USA
[7]Departamento de Ciencias Biológicas, Facultad de Ciencias Naturales, Universidad Icesi, Cali, Colombia
[8]Posgrado en Ciencias Biológicas, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad de México, Mexico
[9]Department of Zoology, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[10]Estación de Biología Chamela, Instituto de Biología, Universidad Nacional Autónoma de México, A.P. 21, San Patricio-Melaque, Jalisco, México
[11]Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045, USA
[12]Twitter: @Luke_Mahler
[13]Lead contact
*Correspondence: christopher.boccia@mail.utoronto.ca (C.K.B.), luke.mahler@utoronto.ca (D.L.M.)
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SUMMARY

Air-based respiration limits the use of aquatic environments by ancestrally terrestrial animals. To overcome this challenge, diving arthropods have evolved to respire without resurfacing using air held between their cuticle and surrounding water.[1–4] Inspired by natural history observations in Haiti (unpublished data) and Costa Rica,[5,6] we conducted experiments documenting routine air-based underwater respiration in several distantly related semi-aquatic *Anolis* lizard species. Semi-aquatic anoles live along neotropical streams and frequently dive for refuge or food,[7–12] remaining underwater for up to 18 min. While submerged, these lizards iteratively expire and re-inspire narial air bubbles—underwater "rebreathing." Rebreathed air is used in respiration, as the partial pressure of oxygen in the bubbles decreases with experimental submersion time in living anoles, but not in mechanical controls. Non-aquatic anoles occasionally rebreathe when submerged but exhibit more rudimentary rebreathing behaviors. Anole rebreathing is facilitated by a thin air layer (i.e., a "plastron," sensu Brocher[13]) supported by the animal's rugose skin upon submergence. We suggest that hydrophobic skin, which we observed in all sampled anoles,[14,15] may have been exaptative, facilitating the repeated evolution of specialized rebreathing in species that regularly dive. Phylogenetic analyses strongly suggest that specialized rebreathing is adaptive for semi-aquatic habitat specialists. Air-based rebreathing may enhance dive performance by incorporating dead space air from the buccal cavity or plastron into the lungs, facilitating clearance of carbon dioxide, or allowing uptake of oxygen from surrounding water (i.e., a "physical gill" mechanism[4,16]).

RESULTS

Underwater rebreathing behavior in *Anolis*

When submerged, *Anolis* lizards develop a thin plastron of air between their skin and the water, giving them a quicksilver appearance (Figures 1A–1C).[5,6,14,15] Many species can "rebreathe" by inflating the plastron with expired air to form a bubble over the dorsal or lateral surfaces of the head, and then reinspiring this air (Figures 1A–1C; Video S1).

To assess the prevalence and respiratory nature of underwater rebreathing in anole lizards, we conducted submergence experiments on a phylogenetically and ecologically diverse sample of 32 *Anolis* species, plus four non-anoline lizards (STAR Methods). We obtained data for several (three or more) adults for a focal set of 20 *Anolis* species, including five semi-aquatic habitat specialists. In the focal set, we observed rebreathing events in at least one individual of 18 species. However, we observed rebreathing in a majority of individuals tested in just eight, including all five semi-aquatics. Sustained rebreathing (five or more re-inspirations in a trial) was yet more restricted. Although sustained rebreathing occurred in at least one individual in most species (12/20), it was observed in most individuals in just four—all semi-aquatic. Semi-aquatic anoles exhibited both rebreathing ($F_{1,18}$, 27.74; $p < 0.001$) and sustained rebreathing ($F_{1,18}$,

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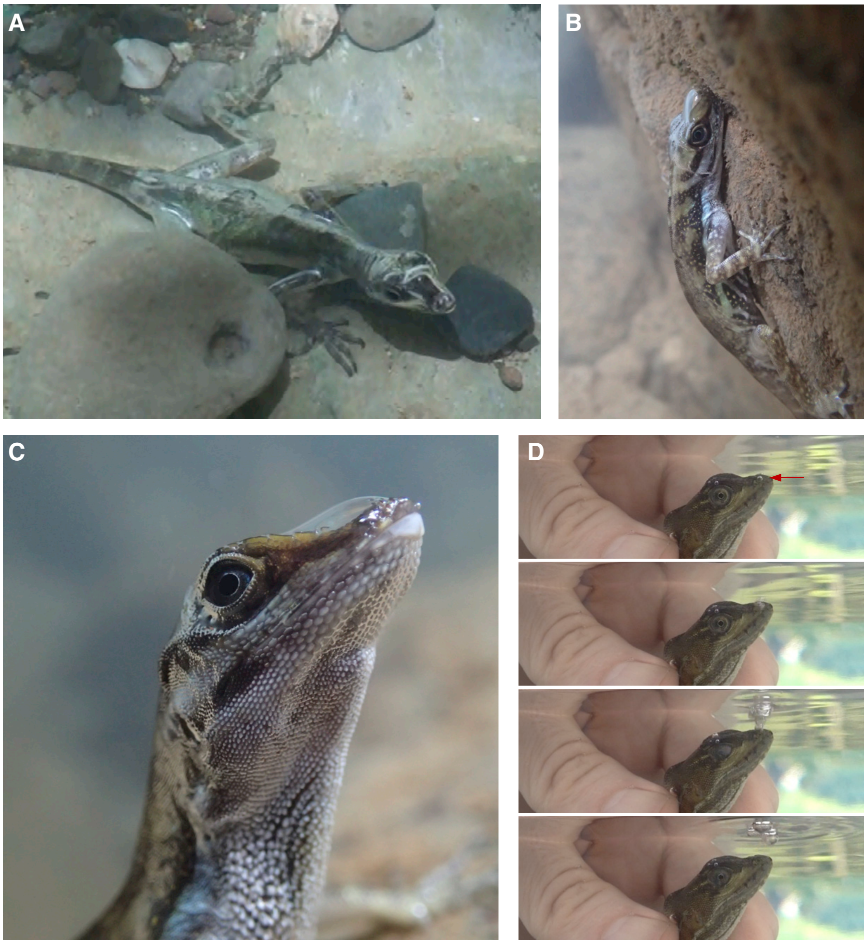

Figure 1. Rebreathing in semi-aquatic anoles

(A–C) *Anolis eugenegrahami* from Haiti (A) and *A. aquaticus* from Costa Rica (B and C) rebreathing underwater in nature. In each, the lizard's hydrophobic skin supports the maintenance of a thin plastron of air around the body. The lizard rebreathes by expiring air from the lungs into this plastron, creating a locally expanded rebreathing bubble, and then re-inspiring this air (see also Video S1).

(D) Non-anoline lizards such as *Basiliscus galeritus* did not form a plastron when submerged. Expired air in these species is lost to the surface as small bubbles, and is thus unavailable for rebreathing. Still video frames (from top to bottom) illustrate this loss of expired air during a single underwater expiration event; the small bubble exhaled over the nares is indicated with a red arrow.

Photo credits: D. Luke Mahler (A and D) and Lindsey Swierk (B and C).

28.18; $p < 0.001$) more frequently than their non-aquatic relatives (Figures 2A and 2B).

Ethogram data collected from trial videos reveal that semi-aquatic anoles rebreathe more during trials ($F_{1,18}$, 67.10; $p < 0.001$; Figures 2C and 2D), rebreathe more frequently ($F_{1,18}$, 31.48; $p < 0.001$; Figures 2G and 2H), exhibit greater species-level performance maxima for number of rebreathing events in a single trial ($F_{1,18}$, 62.00; $p < 0.001$; Figures 2E and 2F), and remain submerged for longer than non-aquatic anoles, controlling for body mass ($F_{1,18}$, 8.60; $p = 0.010$; Figure S2G). We found no relationship between semi-aquatic habitat affiliation and gular movements, another behavior observed during submergence trials ($F_{1,17}$, 0.35; $p = 0.562$; Figures 2I and 2J). Analyses conducted on data bootstrapped using equal-sized species subsamples (three individuals/species) yielded concordant results (Figure S1). Likewise, observations of one or two individuals for 12 additional *Anolis* species corroborate our focal set findings, with sustained rebreathing rarely observed in general (2/12 species) but documented in this sample's sole semi-aquatic representative (*A. eugenegrahami*; Table S1). We observed incidental but not sustained rebreathing in one of four non-anoline lizard species (*Echinosaura horrida*, a semi-aquatic lizard).

Semi-aquatic anoles vary in the anatomical location of bubble formation: *A. aquaticus*, *A. lynchi*, and *A. maculigula* predominantly expired bubbles to a "top of head" position whereas *A. barkeri* and *A. oxylophus* expired to a "side of head" position most frequently (Figure 3). Bubble placement also varied among non-aquatic anoles; we note that five species commonly exhibited vertical narial expirations—a bubble morphology rarely observed in semi-aquatic species (Figure 3). In "top of head" and "side of head" bubble positions, the expanded pocket of expired air often emerged several millimeters posterior or posterolateral to the external nares, demonstrating the contiguity of the plastron, and suggesting an important role of this layer in bubble formation in sustained rebreathers (e.g., Video S1).

Finally, we observed a thin, silvery plastron of air across the body skin surface in every *Anolis* individual we studied, but not in *Basiliscus*, *Echinosaura*, or *Enyalioides* (Figure 1; Table S1).

Oxygen use during underwater rebreathing

To test whether rebreathed air is involved in gas-based respiration, we used a bare fiber microsensor to obtain real-time measurements of oxygen partial pressure (pO_2) from rebreathed air bubbles produced by semi-aquatic anole species (STAR Methods). Rebreathing bubble pO_2 was initially similar to that of ambient air and decreased monotonically during trials (Table S3; Figures 4A–4E and S4), but did not change during simulated inanimate controls (Figures 4F and S3).

Patterns of pO_2 decline were attenuated (i.e., negatively exponential) in a majority of best-performance trials, as predicted if the rebreathing bubble acted as a "physical gill"[16] (Figures 4 and S4; Table S3), although we note that this attenuation is subtle. In such trials, pO_2 half-life (calculated as $\ln(2)/\lambda$, where λ is the exponential decay curvature parameter) was predicted by trial duration, with pO_2 half-life increasing with trial length (half-life duration (s) × s^{-1}, 0.739; R^2, 0.37; $F_{1,12}$, 8.832; $p = 0.012$). Half-life was not predicted by species, ln-transformed mass, or water temperature (Table S3). Patterns for alternative linear

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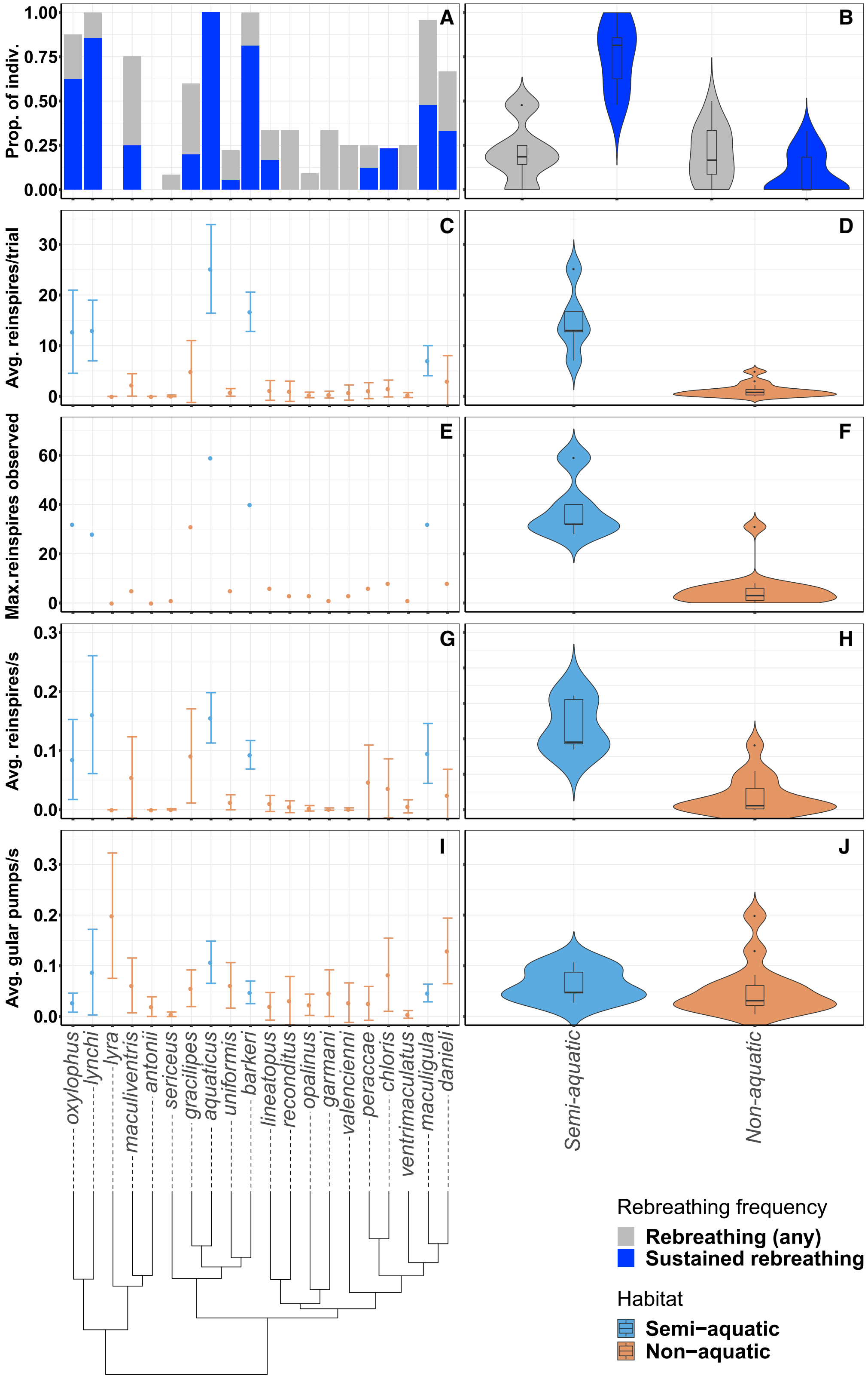

Figure 2. Comparison of rebreathing behavior in semi-aquatic versus non-aquatic anole species

Left panels depict species-level patterns, while right panels compare anole species grouped by habitat or, for the top right plot, by habitat and rebreathing frequency.

(legend continued on next page)

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(rather than exponential) decline models were similar, with longer trials exhibiting shallower slopes (slope, 5.3e−4 hPa × s^{-2}; R^2, 0.39; $F_{1,7}$, 6.12; $p = 0.043$; Table S3) and, additionally, heavier lizards showing slower rates of oxygen decline (slope, 0.077 hPa × s^{-1} × $\ln(g)^{-1}$; R^2, 0.40; $F_{1,7}$, 6.39; $p = 0.039$).

Preferred model fit (exponential versus linear) was not predicted by species, duration, ln-transformed mass, or water temperature (Table S3).

DISCUSSION

Sustained rebreathing as a novel vertebrate respiratory adaptation

Respiration is a key challenge for air-breathing animals in aquatic environments. Although diving arthropods are known to respire using submerged air bubbles,[1,2,19,20] the discovery of underwater rebreathing in a lizard[6] was unexpected. We experimentally confirmed the respiratory nature of this phenomenon—steep, monotonic decreases in the pO_2 of expired and re-inspired air bubbles document respiratory oxygen consumption. The absence of pO_2 decline in inanimate controls demonstrates that observed declines are not an artifact of iterated bubble extrusion.

The observation of regular stereotyped rebreathing in five phylogenetically independent lineages of semi-aquatic anoles, but only rudimentary rebreathing in non-aquatic anoles (Figure 2; Table S1), provides comparative evidence that this difference is likely adaptive.[21] But how might sustained rebreathing increase the fitness of a diving anole?[22]

Performance benefits of rebreathing

If rebreathing enhances performance of underwater activities (e.g., by extending dive duration), its adaptive benefit is clear. Many lizard species dive in response to threats,[23–25] and some will endure extreme hypoxia before resurfacing in the presence of a perceived predator[26] (see also Daniels et al.[27]), depleting glycogen reserves and potentially compromising future escape performance.[28] Others frequently resurface for air and then dive again in the presence of a predator, despite the clear risk of doing so.[29] Such behaviors illustrate the fitness value of extended submergence to diving lizards. Likewise, semi-aquatic anoles readily dive when threatened,[7,8,10,11] and rebreathing may help submerged anoles evade or outwait terrestrial predators. In our experiments, semi-aquatic anoles resurfaced later than non-aquatics (Figure S2).

Rebreathing may also facilitate underwater foraging by prolonging dives, but it remains unknown whether (or how often) semi-aquatic anoles actively search for food underwater. Several species indeed consume aquatic prey,[8–12] but may conceivably locate such prey from a terrestrial vantage, or scavenge deceased aquatic organisms from the shore.[12] Because anoles are visual predators with poor olfaction,[30] it is unlikely they rebreathe to "sniff" for prey underwater, as in some semi-aquatic mammals.[31]

Candidate physiological functions of rebreathing

While the potential for rebreathing to enhance fitness is straightforward, the mechanism by which it may augment dive performance is less clear. We propose several non-exclusive candidate mechanisms.

First, rebreathing may facilitate the incorporation of cranial "dead space" air from the nasal passages, buccal cavity, and trachea into the pulmonary air supply, where it would become available for pulmonary respiration. In reptiles, pulmonary and dead space air volumes are separated by the glottis during apnea;[32] rebreathing would mix these volumes, replenishing depleted pulmonary oxygen and potentially extending dive duration. The value of such mixing may be diminished if anoles are capable of pharyngeal gas exchange (as in some aquatic turtles[33,34]), but to our knowledge this possibility has not been investigated in anoles.

Second, rebreathing might allow diving anoles to access air from the plastron for respiration. All anoles in our study formed a thin plastron of air across their skin when submerged. Rebreathing bubbles were created via narial expansion of this plastron, thus merging the lizard's internal air supply with that of the plastron, and potentially augmenting the oxygen available for respiration during a dive. For plastron air to contribute to respiration would require considerable mixing; whether rebreathing achieves such mixing awaits future study.

Third, rebreathing may clear waste CO_2. In some reptiles, inspiration of CO_2 shortens the duration of breath-holding,[32,35,36] and if CO_2 limits breath-holding in diving anoles, rebreathing could provide a useful CO_2-clearance mechanism. Because CO_2 is highly soluble in water, and because the pCO_2 of submerged respiratory bubbles exceeds that of surrounding water,[4] it quickly clears from such bubbles in diving arthropods.[1] Whether CO_2 clearance is of adaptive value to aquatic anoles will require investigating whether CO_2 limits their diving performance, or whether these lizards are more immediately limited by hypoxia.

Finally, an intriguing potential function of rebreathing is the use of the rebreathing bubble or plastron as a "physical gill" for oxygen uptake from surrounding water, as documented in diving arthropods.[1–4,16] The respiratory function of arthropod physical gills has

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(A and B) Proportion of individuals (A) or species (B) that exhibited rebreathing (at least one re-inspiration event) or sustained rebreathing (five or more re-inspiration events).

(C and D) Average number of re-inspiration events per trial (±2 SEM).

(E and F) Maximum number of re-inspiration events observed in a single trial.

(G and H) Average reinspiration rate (events/s) (±2 SEM).

(I and J) Average gular movement rate (gular pumps/s) (±2 SEM). Summary statistics for species are based on best-performance trials of individuals (those with the greatest number of re-inspiration events, or, if none, individuals' longest-duration trials). Violin plots compare distributions of species values (those depicted in left panels) for semi-aquatic versus non-aquatic anoles; boxes denote median and quantiles, whiskers denote smallest and largest values within 1.5 times the interquartile range; scores outside this distance are denoted as outlying points. The phylogenetic tree shows relationships between species and is derived from Poe et al.[17]

See Figures S1 and S2 for concordant subsampled and trial-averaged results; Table S1 for species-level locality, ecology, and rebreathing trial information; and Table S2 for a rebreathing behavior ethogram.

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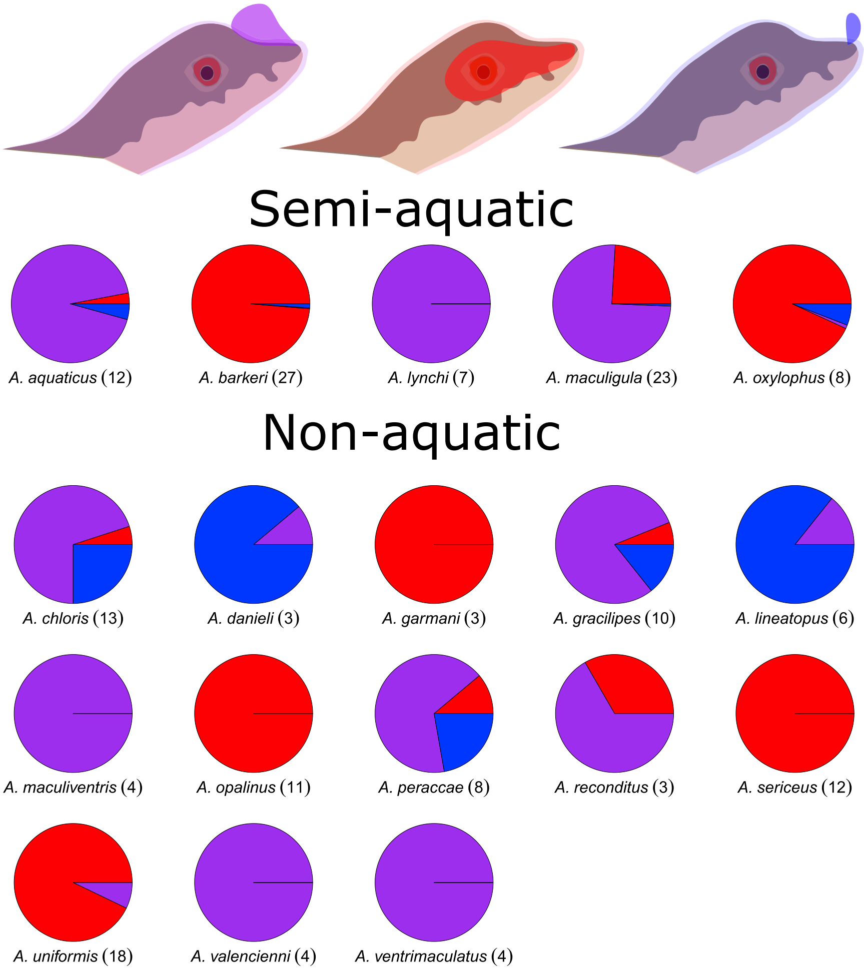

Figure 3. Rebreathing bubble localizations
Proportion of rebreathing bubble localizations observed for all *Anolis* species that met filtering requirements and produced at least one bubble. Colors indicate bubble location on the head (purple, top of head; red, side of head; blue, narial). Numbers of individuals sampled per species are given in parentheses. Illustration credit: Claire Manglicmot.

been studied for more than a century,[1,3,16,37] and recent work has begun to reveal the conditions under which these gills may extend or even indefinitely sustain diving activity. Prolonged underwater respiration is most likely in arthropods that are small, have a low metabolic rate, and can ventilate the gill surface to facilitate gas exchange (e.g., using leg movements or via habitat choice).[4]

Semi-aquatic anoles are larger than most such arthropods, and may thus have relatively little to gain from physical gill gas exchange. Nonetheless, one of the largest diving beetles, *Hydrophilus piceus*, can weigh 2.8 g,[38] which is similar in size to adult female *Anolis lynchi*. Indefinite underwater respiration seems implausible for animals of this size,[4] but the use of a physical gill to at least prolong respiration cannot be ruled out on the basis of size alone. We note that semi-aquatic anoles exhibit a similar relationship between estimated metabolic rate and mass as diving arthropods (Figure 4G). If we assume an insect model for oxygen demand and physical gill oxygen supply,[4] semi-aquatic anoles would be unable to meet their oxygen needs solely via the plastron, but may nonetheless benefit from it by acquiring enough oxygen to extend their dive time, as in large diving insects such as *H. piceus* (Figure 4G). Seymour and Matthews[4] proposed that relatively large animals could improve the efficacy of physical gill respiration by ventillating the plastron surface. Anoles may achieve some ventilation via expiration and re-inspiration, but whether such movement cycles would sufficiently ventilate the boundary layer of water surrounding the plastron is unclear. However, semi-aquatic anoles commonly utilize fast-flowing streams, which may facilitate plastron ventilation.

Although a direct test for physical gill respiration in anoles awaits future experimentation, pO_2 traces from rebreathing bubbles may reveal indirect evidence of such a process. Both theory[16] and empirical work on arthropods[39,40] show that during physical gill respiration, bubble pO_2 should decline following submergence, but that this decline should taper as O_2 lost during respiration is offset by gains from physical gill exchange. Our experiments revealed equivocal evidence for an attenuated pO_2 decline—exponential decay pO_2 models were favored for the best performances of a slight majority of individuals (14/23), but were not favored over a linear decline for the remainder (Table S3). While the former outcomes are consistent with modest physical gill O_2 exchange, a similar pattern may also arise from bradycardia, which could occur during experimental submersion.[23,41] Direct experimentation in a controlled laboratory setting will be required to test whether the pO_2 declines we observed are due to physical gill respiration or changes in metabolic rate during dives.

Exaptation and the repeated evolution of specialized rebreathing

While the sustained underwater rebreathing of semi-aquatic anoles appears to be adaptive, the basic rebreathing ability of anoles may have more prosaic roots. The phylogenetic pattern we discovered suggests a role for exaptation in the evolution of specialized rebreathing.[42] We propose that sustained rebreathing behavior repeatedly evolved in semi-aquatic anoles through adaptive refinement of rudimentary rebreathing abilities that are shared widely across the genus because of an exaptive trait—hydrophobic skin.

An exaptation is an ancestrally inherited trait that has been coopted for a novel adaptive purpose.[21,42,43] We suggest that the thin plastron of air observed in all anoles when submerged[6,15] is an exaptation that permits underwater rebreathing. Since all anoles we submerged developed a plastron, including many that rarely, if ever, occur near water, the plastron is unlikely to

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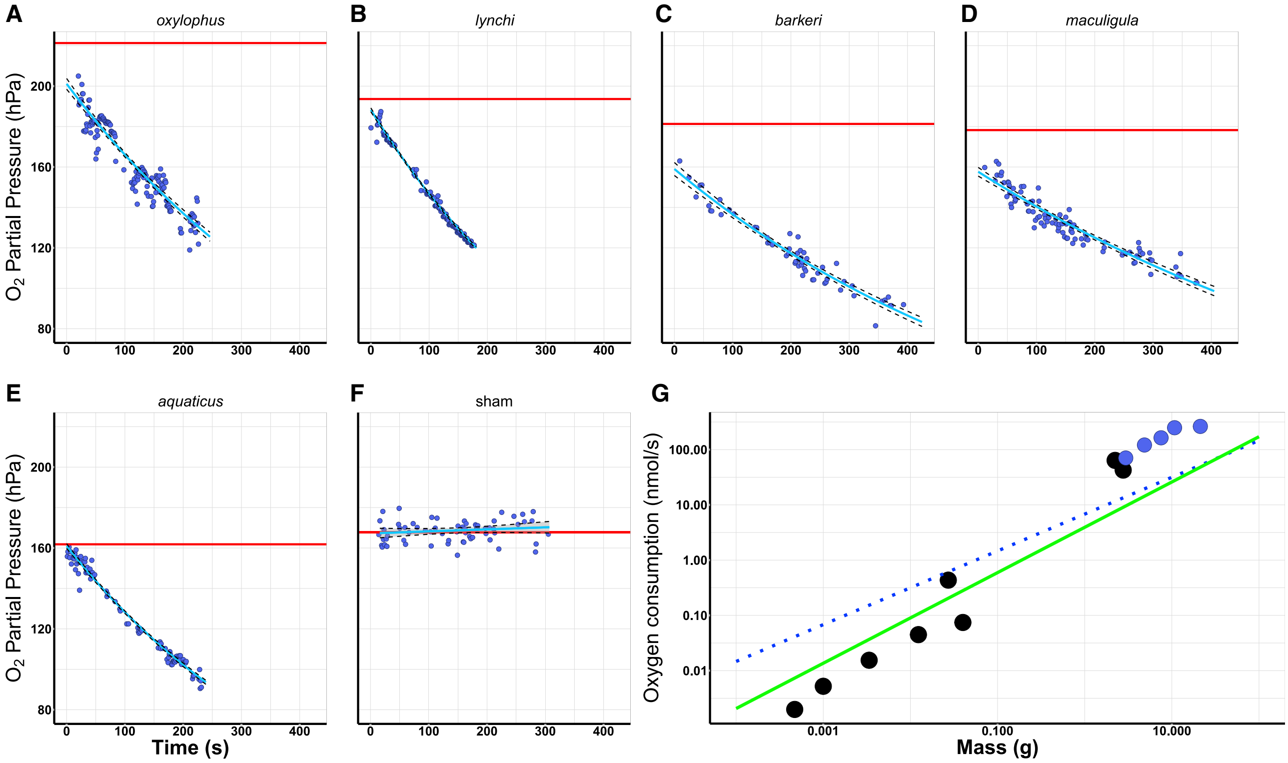

Figure 4. Oxygen consumption patterns

(A–E) Representative plots of partial pressures of oxygen (pO_2) measured in rebreathing bubbles expired and re-inspired by submerged individual semi-aquatic *Anolis* (blue points). Red line shows mean pO_2 measurement for room air; blue line (and dashed CI) depicts fitted exponential decline of pO_2 against time for the period in which the probe was inserted into the rebreathing bubble of a submerged anole. The 95% CI for the exponential model was calculated using the *propagate* R package.[18] Exponential curves had the lowest AIC score for all trials depicted (Table S3). All trials for which we successfully measured bubble pO_2 produced similar relationships (Figure S4).

(F) The "sham" plot shows pO_2 values recorded during a mock submersion in which the exhalation/re-inspiration of diving anoles was mimicked by pumping an air-filled syringe; pO_2 values did not shift from the average room air pO_2 values over a 5 min interval (see also Figure S3).

(G) Plot of oxygen consumption rates against mass for plastron-forming aquatic insect species (black points, from Seymour and Matthews[4]) and semi-aquatic anole species (blue points, this study). Green solid line shows average insect oxygen demand by mass estimated from literature values; blue dotted line shows the oxygen supplementation rate of the plastron assuming a hemispheroid plastron with a 20 μm thick boundary layer; both lines from Seymour and Matthews.[4] Where demand exceeds supplementation, indefinite plastron respiration is not possible; likewise, species with oxygen consumption rates above both lines cannot maintain their oxygen supply indefinitely via plastron respiration.

be an adaptation for diving. More likely, the anole plastron arose as an evolutionary "spandrel"[44]—the byproduct of skin hydrophobicity that evolved for some other purpose (e.g., to shed rainwater or dew,[45] or for anti-fouling via the "lotus effect"[46], as has been shown in geckos[47–49]).

Regardless of original function, an exaptive benefit of hydrophobic skin is the formation of a plastron underwater (Figures 1A–1C), permitting even non-aquatic anoles to occasionally rebreathe if submerged. Because this thin sheath of air covers the entire snout and head, pulmonary expiration creates a relatively flat, stable air bubble that rises at a low angle from the surface of the head in anoles (e.g., Figure 1C) and is likely to remain connected to the plastron rather than breaking off and floating to the surface. By contrast, other examined lizards, including two from closely related genera (*Basiliscus* and *Enyalioides*[50]), did not form a plastron underwater (e.g., Figure 1D). Although some occasionally expired and re-inspired small air bubbles directly above the nares, because these bubbles were not connected to a larger plastron, they rose from the head at very high angles and quickly detached if they grew large (Figure 1D).

If the plastron is an exaptation for rudimentary rebreathing, we suggest it set the stage for subsequent behavioral respiratory adaptation in aquatic habitat specialist anoles. Indeed, anole species that occur exclusively alongside streams and routinely dive to escape predators exhibit significantly more exaggerated rebreathing behaviors than their non-aquatic congeners (Figure 2). The association of regular, sustained rebreathing with specialization on aquatic habitats provides comparative evidence that these behaviors are adaptive for their present role in diving.[21] That they arose from a more rudimentary rebreathing arrangement made possible by the hydrophobic skin shared by all anoles suggests that the specialized rebreathing of semi-aquatic anoles has an exaptive origin.

The replicated origin of sustained rebreathing in semi-aquatic anoles provides insight about the repeatability of evolution when ancestrally similar species independently colonize novel environments.[30,51] The fact that all semi-aquatic anoles that we studied

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exhibited sustained rebreathing suggests a high degree of predictability in anole adaptation. This observation contradicts previous suggestions that semi-aquatic anoles exhibit little convergence[52] but aligns with expectations based on the striking macroevolutionary convergence observed among several largely independent radiations of anoles.[30,53,54] However, despite their similarities, not all dimensions of rebreathing are strictly replicated among semi-aquatic anole lineages. For example, it appears that bubble location (Figure 3), along with some other components of the rebreathing phenotype (Figure 2), may reflect a many-to-one mapping of form to function, in which each semi-aquatic species has evolved one of a limited number of possible phenotypes to achieve the same function of underwater rebreathing.[55]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.04.040.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.K.B. and D.L.M.; Methodology, C.K.B. and D.L.M.; Investigation, C.K.B., D.L.M., R.E.G., L.S., A.M.M., C.A.E., F.P.A.-V., I.L.B., J.B., K.S.T., R.E.M.-G., S.S., and S.O.; Data curation, C.K.B.; Writing – Original Draft, C.K.B. and D.L.M.; Writing – Review & Editing, all authors; Resources, D.L.M., M.d.R.C., A.G., and F.P.A.-V.; Funding acquisition, C.K.B. and D.L.M.; Supervision, D.L.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
Mendeley Data repository (contains all raw data tables)	This paper	https://doi.org/10.17632/kkshkhhnyn.1
GitHub repository (contains code required to generate paper and supplemental material figures and run all analyses)	This paper	https://github.com/chrisboccia/anole-rebreathing-curbio
Experimental Models: Organisms/Strains		
Anolis aquaticus / semi-aquatic, n > 2	Las Cruces Biological Station (LC), Costa Rica, GPS: (8.784142, -82.959552)	N/A
Anolis barkeri / semi-aquatic, n > 2	Los Tuxtlas Research Station (LT), Mexico, GPS: (18.585184, −95.075109)	N/A
Anolis lynchi / semi-aquatic, n > 2	Bilsa Biological Station (BB), Ecuador, GPS: (0.347079, −79.711494); Monterreal Rainforest (MR), Ecuador, GPS: (−0.09246, −78.98974)	N/A
Anolis maculigula / semi-aquatic, n > 2	Parque Nacional Natural Tatamá (TP), Colombia, GPS: (5.227238, −76.082875)	N/A
Anolis oxylophus / semi-aquatic, n > 2	La Selva Biological Station (LS), Costa Rica, GPS: (10.431059, −84.006792)	N/A
Anolis antonii / non-aquatic, n > 2	TP	N/A
Anolis chloris / non-aquatic, n > 2	TP, BB, MR	N/A
Anolis danieli / non-aquatic, n > 2	TP	N/A
Anolis garmani / non-aquatic, n > 2	The Last Resort (LR), Jamaica, GPS: (18.358214, −77.658496)	N/A
Anolis gracilipes / non-aquatic, n > 2	BB, MR, Milpe Bird Sanctuary (MS), Ecuador, GPS: (0.03073, −78.86663)	N/A
Anolis lineatopus / non-aquatic, n > 2	LR	N/A
Anolis lyra / non-aquatic, n > 2	BB, MR	N/A
Anolis maculiventris / non-aquatic, n > 2	BB, MR, MS	N/A
Anolis opalinus / non-aquatic, n > 2	Holywell Recreational Area (HR), Jamaica, GPS: (18.085748, −76.725574); Mr. Bernard's Health Farm (MB), Jamaica, GPS: (18.110008, −76.448441)	N/A
Anolis peraccae / non-aquatic, n > 2	BB, MR	N/A
Anolis reconditus / non-aquatic, n > 2	HR	N/A
Anolis sericeus / non-aquatic, n > 2	LT	N/A
Anolis uniformis / non-aquatic, n > 2	LT	N/A
Anolis valencienni / non-aquatic, n > 2	LR	N/A
Anolis ventrimaculatus / non-aquatic, n > 2	TP	N/A
Anolis eugenegrahami / semi-aquatic, n < 3	Plaisance vicinity, Haiti (PL)	N/A
Anolis antioquiae / non-aquatic, n < 3	TP	N/A
Anolis christophei / non-aquatic, n < 3	Ébano Verde Scientific Reserve (EV), Dominican Republic GPS: (19.03311, −70.54298)	N/A
Anolis duellmani / non-aquatic, n < 3	LT	N/A
Anolis fasciatus / non-aquatic, n < 3	MS	N/A
Anolis fowleri / non-aquatic, n < 3	EV	N/A
Anolis insolitus / non-aquatic, n < 3	EV	N/A

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anolis megalopithecus / non-aquatic, n < 3	TP	N/A
Anolis polylepis / non-aquatic, n < 3	LC	N/A
Anolis princeps / non-aquatic, n < 3	BB	N/A
Anolis purpurescens / non-aquatic, n < 3	TP, BB	N/A
Anolis sagrei / non-aquatic, n < 3	LR	N/A
Basiliscus galeritus / semi-aquatic, n < 3	MR	N/A
Echinosaura horrida / semi-aquatic, n < 3	BB	N/A
Echinosaura apodema / semi-aquatic, n < 3	LC	N/A
Enyaliodes oshaughnesseyi / non-aquatic, n < 3	MS	N/A
Software and Algorithms		
R version 3.6.1 (2019-07-05)	[56]	https://www.r-project.org/
Python 3.7.4	[57]	https://www.python.org/
BORIS	[58]	https://boris.readthedocs.io/
FireSting GO2 Manager	Pyro Science	https://www.pyroscience.com/en/products/all-meters/fsgo2#downloads
Other		
FireSting GO2 Pocket Oxygen Meter	Pyro Science	FSGO2; https://www.pyroscience.com/en/products/all-meters/fsgo2
Bare Fiber Oxygen Microsensor	Pyro Science	OXB50; https://www.pyroscience.com/en/products/all-sensors/oxb50
Retractable Fiber Oxygen Microsensor	Pyro Science	OXR50; https://www.pyroscience.com/en/products/all-sensors/oxr50
GoPro Hero 4	GoPro	HERO4; https://gopro.com/en/ca/update/hero4
Canon EOS Rebel T6	2020 Canon U.S.A.	1159C003AA; https://www.usa.canon.com/internet/portal/us/home/products/details/cameras/eos-dslr-and-mirrorless-cameras/dslr/eos-rebel-t6-ef-s-18-55mm-is-ii-kit
Sony DSC-RX100M5	Sony of Canada	DSC-RX100M5; https://www.sony.ca/en/electronics/support/compact-cameras-dsc-rx-series/dsc-rx100m5

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Christopher K. Boccia (christopher.boccia@mail.utoronto.ca).

Materials Availability

This study did not generate any unique reagents.

Data and Code Availability

The code generated during this study is available at GitHub: https://github.com/chrisboccia/anole-rebreathing-curbio. Original data for figures in the paper are available at Mendeley Data: https://doi.org/10.17632/kkshkhhnyn.1.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We conducted rebreathing assays on adult individuals of 32 species of anoles (six semi-aquatic; 26 non-aquatic), and four non-anoline lizard species (three semi-aquatic; one non-aquatic)—see Key Resources Table, Table S1). Individuals were captured by hand or using pan fishing poles; all lizard handling and experimental protocols were approved by our institutional animal care committees (see Acknowledgments).

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METHOD DETAILS

Sampling

Following an early observation of rebreathing in *A. eugenegrahami* in Haiti in August, 2009, we assessed rebreathing ability from May, 2017 to July, 2019 at various field locations or field stations in Colombia, Costa Rica, the Dominican Republic, Ecuador, Jamaica, and Mexico (see sources column in Key Resources Table). As sampling was based on opportunistic capture of anoles during fieldwork, species varied greatly in numbers of individuals sampled (range: 1-27).

Because of this variation in sampling, and because many species exhibited considerable among-individual variation in behavior, we took two steps to account for sampling error. First, we only included species for which we had sampled three or more adult individuals in quantitative analyses. Our focal species set thus includes 20 *Anolis* species (five semi-aquatic; 15 non-aquatic). We did not include the remaining 12 anole species (one semi-aquatic; 11 non-aquatic) and four non-anoline lizard species (three semi-aquatic; one non-aquatic) in analyses, but summarize the results of these trials in Table S1. Second, we used bootstrap resampling to test whether our results were influenced by among-species differences in sampling, repeating all analyses 10,000 times using three randomly sampled individuals for each species in the focal species set (Figure S1).

Testing for Rebreathing

We assayed rebreathing behavior by observing lizards during voluntary dives (in nature, or in aquaria), during experimental submersion in field laboratory settings, or both. All experimental submersions and laboratory dives took place in a controlled aquatic environment. At each field laboratory, an aquatic arena (bucket, aquarium, or small pool) was filled with clear, unchlorinated, room-temperature water. Ambient air and arena water temperatures varied among sites. In a few trials (n = 14) we successfully induced lizards to voluntarily dive and remain submerged by mimicking predatory movements (typically this occurred when lizards chose to dive rather than swim in response to simulated predatory movements during swimming speed experiments conducted for a related project; unpublished data). In these cases, we observed the lizard's behavior until it voluntarily resurfaced. More often (n = 685 trials), we tested for rebreathing behavior by conducting experimental submersion trials in which we gently grasped anoles around their pelvic girdles and slowly lowered them underwater. Lizards restrained in this manner could easily wriggle free and resurface at any point. Each trial ended when the lizard freed itself and resurfaced, or if it appeared fatigued. A trial was considered successful if the lizard was cooperative (e.g., not immediately wresting free to resurface) and remained submerged for at least 15 s. Submersion trials lasted from 15 s to 12 min, 25 s and individuals that were observed to rebreathe were tested up to five times, with successful trials separated by > 15 min. We note that one *A. barkeri* was observed to remain submerged for greater than 18 min; however, because it hid out of view, this trial was not included in our analyses. All submersion trials were recorded using a submerged waterproof video camera (GoPro Hero 4), or a camera placed above the water or to the side of a clear tank (GoPro Hero 4, Canon EOS Rebel T6, Sony DSC-RX100M5), or both. Following experiments, we measured the snout-to-vent length (SVL) and weight of each lizard and released it at the site of capture.

Oxygen Partial Pressure Measurement

To examine whether rebreathed air is involved in underwater respiration, we tested for a decrease in pO_2 in sequentially rebreathed air bubbles. Where possible, for individual lizards that exhibited long bouts of sustained rebreathing, we measured pO_2 within the rebreathed air bubble throughout the submersion trial with a bare fiber oxygen microsensor (PyroScience OXB50 or OXR50 probe and FireSting GO2 m). During trials, we held the sensor fiber directly above one nostril of the lizard by hand so that it would enter air bubbles iteratively expired and re-inspired during rebreathing (see video of *A. maculigula* in Video S1). The probe logged the partial pressure of oxygen (in hectopascals, hPa) in the air or water surrounding the tip once per second, yielding a pO_2 trace for the duration of the trial. Only pO_2 readings from the air bubble were retained in the trace for later analysis (readings from water were filtered out by sensor signal intensity). Trials shorter than 100 s, with fewer than 10 pO_2 reads, or characterized by excessive measurement noise ($R^2 < 0.75$ in linear regression of pO_2 hPa on elapsed time) were excluded from statistical analyses (excluded reads were visually inspected to confirm that rejection was due to noise rather than assumption of a linear regression model). From the remaining traces, we selected those corresponding to the longest dive for each individual. In the absence of respiration, rebreathed bubble pO_2 is expected to be stable at shallow depths and relatively short periods of time. To rule out the possibility that observed changes in bubble pO_2 could result solely from the physical motions of submerged bubble expansion and retraction, we measured pO_2 in air bubbles produced by repeatedly pumping a qualitatively similar volume of laboratory air using a syringe during simulated underwater rebreathing trials of similar length (n = 5; Figure S3).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of Rebreathing Behaviors

Preliminary observations revealed that some individual lizards did not rebreathe during trials, some did so occasionally and irregularly, and some exhibited frequent underwater rebreathing. We thus first scored each individual both for the presence of "rebreathing" (those re-inspiring air one or more times in any single trial), and for the presence of "sustained rebreathing" (those re-inspiring five or more times in any single trial; note that "sustained rebreathing" is a subset of "rebreathing"). We scored individuals that did not re-inspire during trials as "non-rebreathers."

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Next, for each trial, we collected additional respiratory behavior data from videos using an event-based ethogram approach in BORIS.[58] We logged the following events: trial start, trial end, bubble expiration start, bubble expiration end (due to re-inspiration or release), bubble re-inspiration, bubble release from nares, gular movement (''gular pump''), and start and end times for any period for which the head or gular regions of the lizard were not visible to the camera. We also recorded the locations of expired air bubbles (e.g., top of head, side of head, or directly above the external nares). From these data, for each trial we quantified several potential rebreathing behaviors or performance measures, including total submergence time, the total number of re-inspirations, rebreathing rate (re-inspirations/s), numbers of rebreathing-associated behaviors (gular pumping and bubble releases), and the relative frequencies of alternative bubble locations observed. Expanded descriptions of ethogram events and durations are provided in Table S2. Data were extracted from BORIS output files using a custom Python script (see GitHub repository).

Finally, because of the potential role for an air-filled skin covering (''plastron'') in underwater rebreathing, we scored the subject of each trial for the presence or absence of a silvery plastron covering the body during submersion.

Statistical Analysis of Rebreathing Behaviors

For analysis of respiratory behaviors, we used each individual's best performance trial, defined as the trial with the greatest number of re-inspiration events (analysis of trial means yielded similar results; Figure S2); in cases where there were two or more trials with the same number of re-inspiration events, we used the longest of the tied trials. For individuals that did not re-inspire, we used their longest trial. We used species means of best individual performances as species values for comparative analyses. To test whether rebreathing is a potential adaptation for semi-aquatic habitat affiliation, we fit phylogenetic generalized least-squares (PGLS) linear models, with habitat affiliation (semi-aquatic versus non-aquatic) as a binary predictor variable, and species mean values of rebreathing behavior measurements as response variables (note that for dive duration, we also included mass as a covariate, as dive duration scales with body mass[59]). We accounted for phylogenetic non-independence among model residuals assuming a Brownian motion branch length transformation.[60] We logit-transformed proportions prior to analysis.[61] We conducted all comparative analyses using the ''ape,'' ''caper,'' and ''phytools'' R packages,[60,62,63] and a time-calibrated phylogeny for *Anolis*, pruned to match our sample, from Poe et al.[17]

Testing for ''Physical Gill'' Oxygen Transfer

As a preliminary test of whether rebreathing anoles use the rebreathed air bubble to obtain oxygen from surrounding water, we tested for nonlinearity in the shape of the pO_2 trace, as would be expected if O_2 lost via respiration was offset by exchanged O_2 from surrounding water.[16] For each trace in our filtered sample, we compared the fit of linear versus exponential decay models (using AIC_c in the ''MuMIn'' R package;[64]). We note that alternative mechanisms may also produce nonlinearity in pO_2 decline (e.g., a reduction in metabolic rate during the trial). Conversely, a linear decrease in pO_2 would constitute evidence against the use of a rebreathed air bubble as a physical gill during a trial. For those trials for which an exponential decay was substantially favored ($\Delta AIC > 2$), we then tested whether the exponential rate of decline varied as a function of trial duration, water temperature, species, or ln-transformed individual body mass using linear regression. Note that for one trial (*A. oxylophus ANOX6*), a linear model was favored over an exponential decline because of the inability of the latter model to represent an accelerating, rather than decelerating, decline in pO_2 over time; omitting this trial does not change our results.

For trials for which we could not reject a linear model of oxygen decline, we separately tested for relationships between decline rate and trial duration, water temperature, species (with post hoc differences assessed using a Tukey honest significant differences test), and body mass using linear regression.

We tested if preferred model fit (linear or exponential) for each individual's best trial varied by species, ln-transformed mass, or trial duration using a binomial family generalized linear model with a logit link.

Finally, we calculated oxygen consumption based on our observed O_2 partial pressure loss over time and assuming the lung volume:mass ratio for a related iguanian lizard (*Iguana iguana*) reported by Perry.[65]