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Novel rebreathing adaptation extends dive time in a semi-aquatic lizard

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Bubble use evolved in many small invertebrates to enable underwater respiration, but, until recently, there has been no evidence that vertebrate animals use bubbles in a similar manner. Only one group of vertebrates, semi-aquatic *Anolis* lizards, may be an exception: these lizards dive underwater when threatened and, while underwater, rebreathe a bubble of air over their nostrils. Although it seems that rebreathing should be adaptive, possibly functioning to extend the time that lizards remain in underwater refugia, this has not been empirically tested. Here, I demonstrate that rebreathing serves to extend dive time in a semi-aquatic anole, *Anolis aquaticus*. I prevented the formation of normal rebreathing bubbles by applying a commercial emollient on the skin surface where bubbles form to assess the impact of bubbles on rebreathing cycles, gular pumps, and dive times. Lizards that were allowed to rebreathe normally remained underwater an average of 32% longer than those with impaired rebreathing, suggesting a functional role of rebreathing in underwater respiration. Unlike rebreathing, gular pumping was unaffected by treatment and may warrant further research regarding its role in supplementing underwater respiration. This study provides evidence that vertebrates can use bubbles to respire underwater and raises questions about adaptive mechanisms and potential bio-inspired applications.

1. Introduction

Many air-breathing animals have evolved novel uses for air bubbles to address physiological and ecological challenges when underwater. Although bubbles are used by air-breathing animals in a range of unusual underwater applications (e.g. olfaction [1–3], communication [4], thermoregulation [5], access to mates [6] or long-distance travel [7]), one use of bubbles that particularly captures attention is the use of bubbles for underwater respiration. Several small invertebrates can attach bubbles of air to their hydrophobic body surfaces (e.g. insects [8–11]; scorpions [12]) or their hydrophobic extended phenotypes (e.g. spiders [13–15]; aphids [16]) when underwater. These bubbles act as air storage for respiration (e.g. [9,17,18]) and, remarkably, sometimes as a 'physical gill' [19,20] that allows air-breathing arthropods to obtain O_2 and expel CO_2 through the bubble's gas exchange with the surrounding water (as reviewed by [21,22]).

No air-breathing vertebrate was suspected to use bubbles for underwater respiration until the recent documentation of rebreathing behaviour in Neotropical semi-aquatic *Anolis* lizards [23–26]. Upon diving, a semi-aquatic anole exhales a bubble of air that clings to the top (figure 1*a*) or sides (figure 1*b*) of its head, sometimes joining with existing air pockets on the body surface. The bubble is then 'rebreathed' at regular intervals during a dive (electronic supplementary material, video S1); high-speed macro videos suggest that scale and head morphology helps facilitate the movement of

air from the body surface to the nostrils (unpublished data), permitting the bubble to appear and disappear around the head with every respiratory cycle. Semi-aquatic anoles have long hair-like microscopic spinules on the skin trapping air and reducing wettability [27], allowing air to be retained on the body surface during dives. In addition to rebreathing bubbles while diving, anoles can also visibly extend, then retract, the hyoid (consisting of thin, mostly cartilaginous rods in the buccal floor) to create positive pressure to pump air from the gular (throat) region to the lungs, known as 'gular pumping' [28].

The function of rebreathing bubbles is unknown but is hypothesized to facilitate longer dives, as bubble air loses P_{O2} as anoles rebreathe over time compared to non-rebreathing controls [25]. Although other non-aquatic anole species also have hydrophobic skin and may produce small bubbles if placed underwater (e.g. [29]), semi-aquatic anoles more consistently use sustained rebreathing behaviour, rebreathe more often during each dive, and remain underwater longer than do non-aquatic anoles [25]. Rebreathing behaviour, along with related semi-aquatic morphologies, swimming speed, and skin microstructures, together suggest that these are semi-aquatic adaptations that have repeatedly evolved in the *Anolis* genus and demonstrate convergence [25–27]. Given the observations of semi-aquatic anoles diving underwater when threatened, e.g. [23,30], it follows that this convergent rebreathing behaviour during dives may allow semi-aquatic anoles to extend time spent in underwater refugia away from predators. However, this or any other potential fitness-enhancing function of rebreathing bubbles is yet to be documented.

Using a representative semi-aquatic anole, *Anolis aquaticus*, I experimentally prevented rebreathing bubbles from being formed to examine the effect of rebreathing on dive time. *A. aquaticus* is found in lowland and premontane wet forests in southwestern Costa Rica and western Panama [31], nearly always in habitats next to small and rocky streams. This small lizard (up to 71 mm in snout-vent length) is hunted by various birds and snakes [32] and uses both camouflage [33,34] and diving [23,30] to avoid predators. *A. aquaticus* consistently rebreathes during dives, which can last at least 16 minutes [23] and likely longer. Its body surface is also very hydrophobic (unpublished data). To examine if rebreathed bubbles lengthen dive time, I applied a commercial emollient to *A. aquaticus* skin surfaces where bubbles form to block the path of underwater air movement between the body surface and the nostrils, while leaving the nostrils unobstructed. This prevented normal rebreathed bubbles from being formed but permitted anoles to otherwise dive and resurface naturally. I compared dive lengths in *A. aquaticus* with and without the ability to produce normal bubbles, along with bubble production (if any) and gular pumping rates in each group, to test whether semi-aquatic anoles use rebreathed bubbles to increase their capacity for long dives. This study is the first step to determining the functional role of respiratory bubble use in air-breathing vertebrates.

2. Methods

Anolis aquaticus (*n* = 30; 24 males and 6 females) were collected by hand or lasso from the boulders, banks, and vegetation bordering the Rio Java at Las Cruces Biological Station, Puntarenas Province, Costa Rica, in 2021 and 2022. Upon capture, lizards were immediately placed into a cotton drawstring bag and transported a short distance to the location of the field arena (less than a 5-min walk from the site of collection). The arena was a clear plastic tank (74 × 45 × 34 cm; L × W × H) filled with fresh stream water to approximately 30 cm, containing several stream rocks and a single flat rock used as an underwater lizard perch. An action camera (EK7000Pro, Akaso, Frederick, MD, USA) was placed underwater in the arena *ca* 30 cm from the designated perch to record each trial. The entire arena was securely placed in the water at the stream edge, and tank water was replaced immediately before each trial.

Lizards were randomly assigned to either the impaired bubble treatment group or the normal bubble control group. In the treatment group, a thin layer of commercial emollient (Honest Beauty Daily Moisturizer, Honest Company, Los Angeles, CA, USA) containing hydrophilic humectants was evenly applied with a soft paintbrush to the upper snout and both lateral sides of the head (figure 1c), avoiding the nostrils and eyes; similar water-based substances (typically containing prescription ingredients) are frequently used to treat skin conditions in herpetofauna, e.g. [35]. The control group received an application of water, instead of the emollient, also applied using a paintbrush. Immediately after applying the emollient or water, the lizard was submerged into the arena and was allowed to grasp the underwater perch autonomously. Lizards (*n* = 2) failing to grasp the perch autonomously when initially submerged were excluded from trials. All researchers retreated behind a blind *ca* 3 m from the arena; from the blind, lizard dive length was measured with a stopwatch and the presence/absence of rebreathing was noted. As soon as the lizard resurfaced, the total dive length (s) was recorded, and the trial was concluded. Body mass (g) and sex were noted for each lizard. Any residual emollient was removed from lizards in the treatment group using a cotton swab, and each lizard was released at its site of capture after receiving a temporary marking (a single drop of nail polish on the base of the tail) to prevent reuse; study locations within the same river shifted *ca* 0.4 km between years, and *A. aquaticus* average home ranges are 21 m² (M. Delfin & L. Swierk, unpublished data), making it unlikely that lizards were used more than once.

Videos of trials were reviewed to obtain counts of rebreathed bubbles and instances of gular pumping. A bubble was considered 'rebreathed' if it was exhaled while clinging to the lizard's body surface (typically the dorsal surface of the head) (figure 1d) and then re-inhaled to the point where it was no longer visible; this full cycle was considered to be a single rebreathed bubble. A single gular pump included the extension (figure 1e) and full retraction (figure 1f) of the hyobranchium.

(a) Statistical methods

To test if dive length was influenced by rebreathing, a multiple regression model was used with group (control *v.* treatment), body mass (g) and year as predictors. The relationships between dive length and the counts of rebreathed bubbles or gular pumps were tested using two generalized linear models (family = quasipoisson). In these two models, either the count of
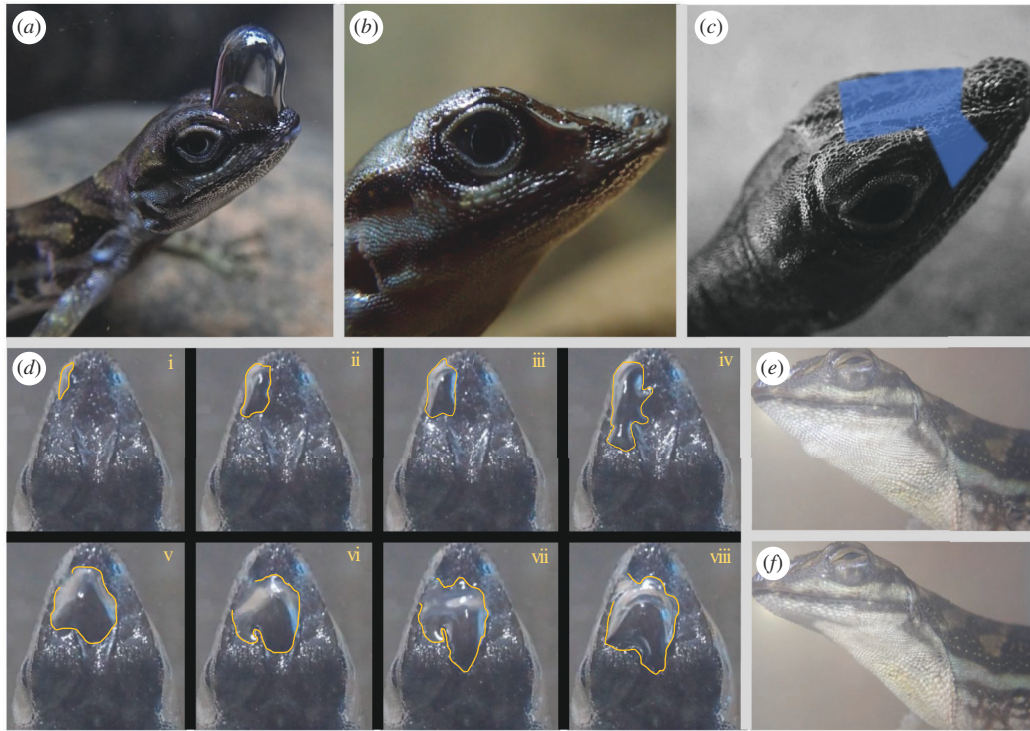
Figure 1. *Anolis aquaticus* rebreathing air bubbles located (*a*) dorsally on the snout and (*b*) laterally between the eye and nostril. (*c*) Time progression of the formation of single dorsal bubble, with 0.2 s elapsed between each image i–iv, and 0.07 s elapsed between each image iv–vii. The bubble is held briefly, slightly expanding (vii–viii; 2.0 s) and is then rebreathed. (*d*) Area (blue shading) of application with emollient (impaired bubble treatment group) or water (normal bubble control group). Gular pumping shown at (*e*) its greatest extension followed by (*f*) contraction.

rebreathed bubbles or the count of gular pumps was the response variable, and group, body mass, year and dive length were predictors; two complementary linear regression analyses similarly modelled the (log+1 transformed) rates of bubble rebreathing (# bubbles/min) and gular pumping (# pumps/min) with group, body mass and year as predictors. Sex was initially included in all models, but it did not significantly improve fit and was removed according to standard model selection procedures [36,37]; no model qualitatively differed after sex was excluded. The *emmeans* package [38] was used to determine the estimated marginal means (EMMs) and effect size of treatment in the multiple regression model.

Values of *p* were obtained for individual predictors using the Anova() function in the *car* package [39], using *F*-tests in the two generalized linear models to account for overdispersion. The residuals of all models were inspected using diagnostic plots, and model appropriateness was confirmed [40]. Statistical tests maintained an alpha of 0.05, were two-tailed and were performed in R (v. 3.6.3; R Development Core Team 2020).

3. Results

A total of 28 trials (control = 15, treatment = 13) using *A. aquaticus* adults (*n* = 15; 8 control, 7 treatment) and subadults (*n* = 13; 7 control, 6 treatment) were completed. In the control group, all lizards rebreathed large bubbles (with an area of adherence slightly smaller than the anterior head and snout surface). Some (*n* = 6) treatment group lizards infrequently rebreathed *ca* 1 mm bubbles that appeared directly over the individual nostrils but did not adhere to the surface of the skin (similar to the 'partial' or 'narial' bubbles produced by most non-aquatic anole species, see [25]); a Wilcoxon rank sum exact test confirmed no difference in dive length (W = 16, *p* = 0.534) or gular pumping rate (W = 24, *p* = 0.125) between treatment group lizards with and without 'partial' bubbles. One control group lizard with an especially high dive time (477 s) was determined to be an outlier (G = 3.259, U = 0.646, *p* = 0.005) using the *outlier* package [41] in R, and this observation was removed from subsequent analyses.

The multiple regression model that tested the factors influencing dive length was significant overall ($F_{3,23}$ = 3.669, *p* = 0.027), with lizards in the control group remaining underwater longer than those in the treatment group (table 1; figure 2*a*). Effect size analysis classified treatment as having a 'large' effect on dive duration (Cohen's *d* = 0.958 ± 0.419 s.e.; [42]), with control lizards diving on average 67.5 s longer than treatment lizards (figure 2*b*). The final model also suggested a non-significant trend of mass increasing with dive time; year was unrelated to dive time (table 1).

Treatment affected a lizard's number of rebreathed bubbles but not the number of gular pumps (table 2). Control lizards rebreathed an average of 6.03 ± 0.87 s.e. (normal) bubbles per minute, whereas treatment lizards rebreathed an average of 1.80 ± 0.70 s.e. (partial) bubbles per minute (figure 2*c*). Rebreathing rates significantly differed by group in both the generalized linear model (table 2) and also in a complementary linear regression analysis that modelled bubble rebreathing rate as the result of treatment group ($F_{1,21}$ = 18.735, *p* < 0.001), mass ($F_{1,21}$ = 6.241, *p* = 0.020) and year ($F_{1,21}$ = 1.490, *p* = 0.235). In contrast, lizards in both groups produced gular pumps at approximately the same rate (3.45 ± 0.66 s.e. *v.* 3.58 ± 0.85 s.e. gular pumps per minute, control versus treatment lizards, respectively; figure 2*d*); this result was found in both the generalized linear model and in a
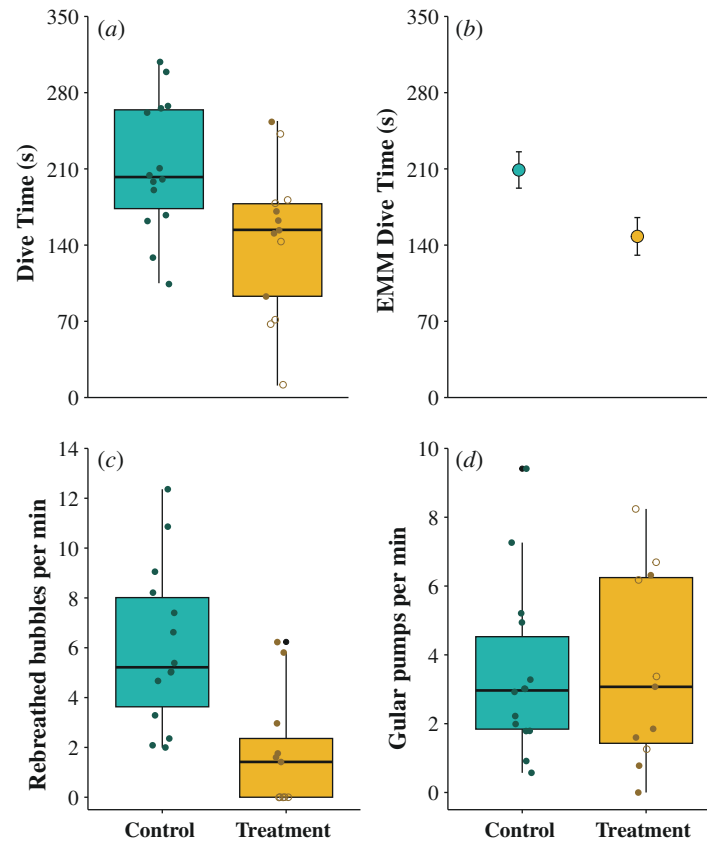
Figure 2. Comparison of normal bubble control (C) and impaired bubble treatment (T) group (*a*) dive times, (*b*) EMMs of dive times accounting for variability in mass and year, and counts of (*c*) rebreathed bubbles and (*d*) gular pumps performed per minute in *A. aquaticus*. Filled blue circles (control group) indicate normal-sized rebreathing bubbles, filled gold circles (treatment group) indicate partial bubbles and open gold circles (treatment group) represent lizards entirely without bubbles.

Table 1. Multiple regression model results of the factors influencing dive length in diving *A. aquaticus*, including treatment group (normal bubble control *versus* impaired bubble), mass (g) and year. Statistical significance ($p < 0.05$) is indicated in bold with an asterisk (*).

factor	estimate	d.f.	sum sq	F	p
treatment	-60.63 ± 24.96	1	23 640	5.898	**0.023***
mass	11.64 ± 6.47	1	12 954	3.232	0.085
year	6.16 ± 25.70	1	231	0.058	0.812

linear regression with gular pump rate against treatment group ($F_{1,21} = 0.001$, $p = 0.975$), mass ($F_{1,21} = 1.953$, $p = 0.177$) and year ($F_{1,21} = 0.166$, $p = 0.687$). Gular pumps were correlated with dive length in both treatment and control lizards (figure 3*a*). Overall, the number of rebreathed bubbles was not related to dive length in our original model (figure 3*b*), though removing treatment from the group from the model revealed a significant correlation between rebreathed bubbles and dive time ($F_{1,23} = 7.628$, $p = 0.011$). Greater body mass corresponded to fewer rebreathed bubbles produced, though body mass did not correlate with gular pump production rate. Full model results are shown in table 2.

4. Discussion

Semi-aquatic anoles are the only group of vertebrates known to rebreathe bubbles of air while underwater (besides when used for olfaction [1–3]), and the only vertebrates in which rebreathed bubbles have a putative respiratory function. In this study, I demonstrate that rebreathed bubbles extend dive time in a semi-aquatic anole, *A. aquaticus*. This finding provides evidence of the functional respiratory contribution of rebreathing, giving context to the observation [25] that O_2 is extracted from rebreathed bubbles by anoles. Preventing rebreathing shortens anole dive time in trial arenas by an average of *ca* 32%; this difference may be even greater in natural streams, when there can be an imminent threat of predation and dives tend to be longer [23,25]. These results provide strong support for the 'scuba tank' hypothesis of anole rebreathing [23], in which the surplus air carried inside and on the hydrophobic body surface of semi-aquatic anoles works like a human diver's scuba tank to extend dive time by providing an additional volume of accessible air. Without rebreathing, much of this additional air is unavailable to anoles, and dives are shortened.

Application of the treatment not only reduced dive time but also reduced rebreathing rates, such that treatment-group *A. aquaticus* in this study had similarly low rebreathing rates to that of non-aquatic anoles (electronic supplementary material, table
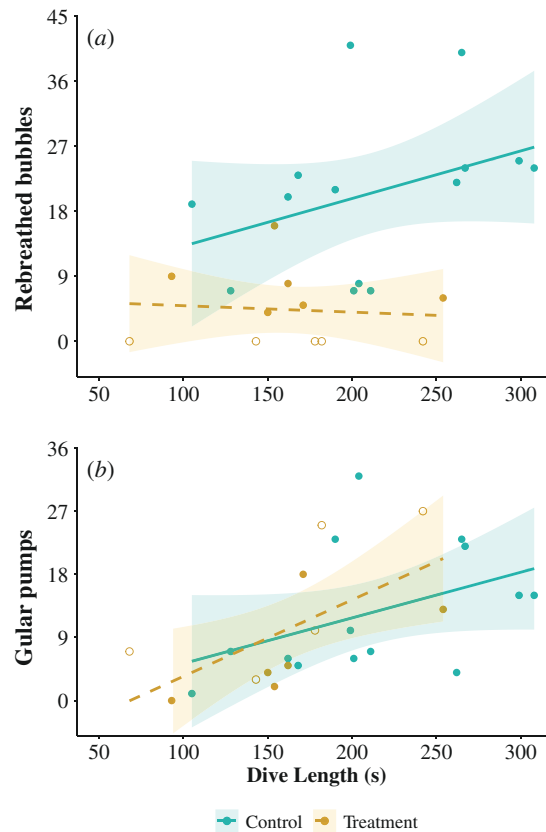
Figure 3. Numbers of (*a*) rebreathed bubbles and (*b*) gular pumps in *A. aquaticus* in the normal bubble control (blue, solid line) and impaired bubble treatment groups (gold, dotted line). Shaded coloured regions around each line of best fit are 95% confidence bands. Filled blue circles (control group) indicate normal-sized rebreathing bubbles, filled gold circles (treatment group) indicate partial bubbles and open gold circles (treatment group) represent lizards without bubbles. Rebreathing, but not gular pumping, rates differed by treatment group.

Table 2. Generalized linear model (family = quasipoisson) results of the numbers of rebreathed bubbled and the numbers of gular pumps produced by diving *A. aquaticus* in normal bubble control and impaired bubble treatment groups. Statistical significance ($p < 0.05$) is indicated in bold with an asterisk (*).

factor	rebreathed bubbles					gular pumps				
	estimate	d.f.	sum sq	*F*	*p*	estimate	d.f.	sum sq	*F*	*p*
treatment	-1.21 ± 0.36	1	64.270	14.044	**0.001***	0.17 ± 0.32	1	1.539	0.257	0.617
dive length	$<0.00 \pm 0.00$	1	16.013	3.499	0.076	0.01 ± 0.00	1	26.040	4.359	**0.049***
mass	-0.14 ± 0.06	1	22.160	4.842	**0.039***	0.05 ± 0.09	1	1.603	0.268	0.610
year	0.64 ± 0.29	1	23.971	5.238	**0.033***	0.32 ± 0.35	1	5.211	0.872	0.361
model	$R^2 = 0.838$					$R^2 = 0.492$				

S1) and produced only the 'partial' or 'narial' rebreathed bubbles that are typical of non-aquatic anoles upon submergence. In effect, by preventing rebreathing, *A. aquaticus* demonstrated a behavioural (diving) phenotype similar to non-aquatic anoles. From a phylogenetic perspective, it seems unlikely that rebreathing represents a key innovation, given the probable multiple independent occurrences of semi-aquatic *Anolis* species evolution [43,44]. Nevertheless, the adaptive function of rebreathing, as identified in this study, could drive the apparent convergence in rebreathing among independently evolved semi-aquatic anoles.

Lizards primarily respire using costal aspiration, a primitive form of aspiration in amniotes lacking accessory respiratory muscles (e.g. a diaphragm), where inspiration and expiration are controlled by costal (axial) muscles (as reviewed by [28]). As these axial muscles are also implicated in lizard locomotion, there can be axial constraints on lizard respiration during periods of strenuous or sustained exercise [45,46]. Unlike rebreathing, *A. aquaticus* gular pumping rates were similar in both treatment and control groups (figure 2*d*), and the numbers of gular pumps were correlated with dive length in both groups (figure 3*b*). Gular pumping is an ancestral trait among lizards [28] that, in some species, may mitigate axial respiratory constraints by providing a supplementary air source to the lungs during periods of exercise (*Varanus* lizards [45]), at high temperatures (*Uromastyx aegyptius* microlepis [47]) or following a long dive and bradycardia (in marine iguanas, *Amblyrhynchus cristatus*, which do not rebreathe [48]).

While semi-aquatic *Anolis* lizards are unlikely to experience locomotion-related respiratory constraints while inactive and rebreathing underwater, they do face respiratory constraints as diving and air-breathing animals. When underwater, gular pumping cannot push new air into a rebreathing anole's lungs, but gular pumping may nevertheless enhance the circulation

of air from the gular/tracheal/nasal spaces past the glottis and into the lungs. By exchanging stored volumes of air and making them available for respiratory use, gular pumping may in fact function in much the same way as a rebreathed bubble itself, as an air redistribution mechanism. Gular pumping has not yet been documented to have any functional role in *Anolis* respiration, either in air or underwater, so this remains to be tested. The maintenance of gular pumping in both treatment and control groups, in addition to other unmeasured variables like bradycardia, may help explain how semi-aquatic anoles may dive, albeit for shorter durations, even if rebreathing is not possible.

An early report [49] suggests that the gular/buccal cavity itself may serve as a respiratory surface, with up to 10% of total CO_2 waste expelled by gas exchange through capillaries below the epithelium of the pharynx and the oesophagus. Though more rigorous tests would clarify how widely applicable this phenomenon is, this mechanism could give insight into how gular pumping may facilitate diving behaviour in semi-aquatic anoles and other air-breathing reptiles. This idea is particularly intriguing if costal aspiration and gular pumping also work together with the potential air–water gas exchange of bubbles to expel CO_2, possibly indicating a three-tiered gas exchange system in semi-aquatic anoles. Additionally, costal aspiration and gular pumping together could directly supplement blood oxygenation if O_2 is exchanged between bubbles and water (as a physical gill [21,22]), though physical gill action alone is unlikely to support long-term underwater respiration given the larger size and greater metabolic demands of vertebrates compared to that of invertebrates, see [22]. Whether bubble air–water gas exchange in vertebrates provides any functional respiratory contribution is yet to be determined. However, gas exchange can occur directly from water to blood vessels in reptiles: for example, some turtles use cloacal or other cutaneous respiration (e.g. [50]), and sea snakes (*Hydrophis* spp.) potentially use cutaneous respiration to oxygenate blood vessels on the head [51].

Many outstanding questions remain regarding the underlying mechanisms of dive extension via rebreathing. In particular, does rebreathing act to merely redistribute volumes of air in and around an anole's body, or are there mechanisms for additional gas exchange with the water through a physical gill-type action? Furthermore, the environment should variably alter rebreathing effectiveness, as stream water flow and level of dissolved oxygen could affect gas exchange with bubble air and thus dive duration (A. Martin, D. Cordero, LS, unpublished data). While some material properties of semi-aquatic anole skin that permit rebreathing have been identified [27], there is substantial potential for bio-inspired innovations relating to hydrophobicity and for the development of underwater technologies (e.g. [52–54]). Given the repeated evolution of rebreathing within the *Anolis* genus, and the repeated evolution of bubble use more broadly among air-breathing animals across taxa, underwater respiration is an emergent focus in studies of adaptability and evolution to novel habitats.

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