

# Effects of Lung Reduction on Rheotactic Performance in Amphibian Larvae

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**ABSTRACT** In this study we test the hypothesis that lung reduction in amphibians is a larval adaptation to reduce buoyancy and facilitate rheotaxis in lotic environments. Larvae of the spotted salamander *Ambystoma maculatum* (family Ambystomatidae) were raised from eggs with (= control) and without (= experimental) access to the air-water interface. The experimental larvae developed significantly smaller and more solidified lungs than the controls. Although mean snout-vent length did not differ between the control and experimental groups, the latter had a significantly greater mean body mass, indicating greater specific gravity in experimentals versus controls. To evaluate rheotactic behavior, larvae were subjected to a water current in an artificial stream. The distance and time required to establish a purchase on the stream bottom were recorded. Although the times did not differ, the distances displaced downstream were significantly less in experimental than in control larvae. We conclude that lung reduction and solidification reduce buoyancy and enhance rheotactic ability in salamander larvae. These findings suggest that lunglessness in plethodontid salamanders evolved as an adaptation to stream environments. © 1994 Wiley-Liss, Inc.

Among vertebrates, evolutionary reduction or loss of lungs has occurred in many stream-dwelling amphibians, including larval frogs and salamanders (Wake, '66; Duellman and Trueb, '86; Wassersug and Heyer, '88). The largest family of salamanders, the Plethodontidae, consists entirely of lungless species and reputedly evolved in mountain streams (Dunn, '26; Wake, '66). Wilder and Dunn ('20) proposed that lunglessness in plethodontids is an adaptation to reduce buoyancy in streams and facilitate locomotion on the stream bottom. This hypothesis has been criticized recently by Ruben and Boucot ('89), Reagan and Verrell ('91), and Ruben et al. ('93). These authors have argued that lunglessness evolved in ancestral plethodontids in terrestrial rather than aquatic environments. Beachy and Bruce ('92), however, have suggested that plethodontid lunglessness is a larval adaptation that serves to reduce downstream displacement in species having lengthy larval periods.

The objective of our research was to test the Wilder-Dunn hypothesis by evaluating the rheotactic behavior of salamander larvae with experimentally reduced lungs. Inasmuch as plethodontids lack lungs altogether, the experiment involved lar-

vae of the spotted salamander *Ambystoma maculatum* (family Ambystomatidae). Aquatic salamanders, like the larvae of *A. maculatum*, may use cutaneous, branchial, and pulmonary gas exchange, but the former two often supply most of the animal's respiratory needs (Whitford and Sherman, '68; Guimond and Hutchison, '72; Ultsch and Duke, '90). Lungs also function as hydrostatic organs that provide buoyancy and allow the animals to adjust their position in the water column (Whipple, '06). Since it has been shown that access to air is required for normal lung inflation and development in other amphibian larvae (Schmalhausen, '87; Pronych, '92), we predicted that larvae of *A. maculatum* denied such access would develop smaller lungs. To test this assumption we raised *A. maculatum* larvae with (= control) and without (= experimental) access to air.

We designed behavioral tests that were predicated on the anticipated results of the larval rearing protocols. Specifically, we postulated that the experimental larvae would be better able to withstand the effects of a stream current. This hypoth-

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esis was tested in trials in which experimental and control larvae were subjected to the force of a water current in an artificial stream. The ability of larvae to establish and maintain a position on the stream bottom, facing the current, is referred to as the rheotactic response. Following the behavioral trials we measured body sizes, lung dimensions, and lung histology in order to evaluate the results of the rearing experiment and to determine the relationships among body mass, lung development, and behavior.

## MATERIALS AND METHODS

Egg clutches of *A. maculatum* were collected in the field in March 1992 from ponds near Highlands, North Carolina. Single eggs were placed in plastic boxes that had been modified as cages by providing screen-covered openings on two opposing sides and on the lid. Two cage sizes were used: short (95 × 95 × 64 mm) and tall (85 × 85 × 120 mm). All of the cages were held in 492 liter aquaria that were provided with cooling units, circulators, and filters. The short cages were completely submerged, denying the larvae access to air, whereas the tall ones projected above the surface. Water temperatures varied between 12° and 16°C. Larvae hatched in late March and were maintained until June 22–23, just prior to metamorphosis, when the behavioral experiments were conducted.

The behavioral trials were conducted in an artificial fiber glass stream 10 m in length by 0.5 m in width. The stream was bedded with sand and gravel from a nearby natural stream, and water from the latter was pumped through the artificial stream under conditions of partial recirculation. Water depth was maintained at approximately 2 cm. The flow rate was 57 l/min. Larvae were selected at random from the two treatment groups and tested in blind trials. For each trial a larva was transferred from its cage to a plastic cup and released gently into the artificial stream 2 m below the upstream end. The downstream location where the larva stabilized its position on the stream bottom for a count of 5 sec was marked. We recorded

the distance from the release point and the time from release to the end of the stabilization period. Twenty larvae from each treatment were used, and each larva was tested three times.

Immediately after the behavioral trials we measured snout-vent length to the nearest 0.1 mm and wet weight to the nearest 0.001 g on living animals that had been anesthetized in MS-222. The larvae were then killed by prolonged immersion in the anesthetic and were subsequently fixed in 7% buffered formalin. Snout-vent lengths and lung dimensions were measured after fixation under conditions blind to treatment. Lung length and width were measured by flattening the lung, and lung surface area was calculated from these measurements; values given are totals for the left and right lungs. Lung tissue was examined using standard paraffin histology (hematoxylin and eosin staining).

Statistical tests were performed using SYSTAT version 5.2.1 on an Apple Macintosh computer. All tests were evaluated at  $\alpha = 0.05$ .

## RESULTS

In conducting the behavioral trials, larvae were released into a current in the artificial stream where they typically swam and tumbled downstream until they settled on the gravel, facing upstream. Since there was no obvious trend associated with trial number, we averaged the time and distance values of the three trials for each of the 20 individuals per treatment category (Table 1). We analyzed the distance and time data separately using Mann-Whitney U-tests. Although there was no significant difference between treatments in time ( $U_s = 205$ ,  $P = 0.892$ ), there was a significant difference in distance ( $U_s = 277$ ,  $P = 0.037$ ); the experimental larvae traveled shorter distances than the controls before establishing a purchase on the bottom. Of the two factors, distance is considered more important than time, because it is the direct measure of displacement by the water current, which, in theory (Wilder and Dunn, '20; Beachy and Bruce, '92), the animal seeks to minimize or control.

TABLE 1. Results of behavioral trials of *Ambystoma maculatum* larvae<sup>1</sup>

Treatment	No. of larvae	Mean distance (m) displaced per individual			Mean time (sec) to stabilization per individual		
		Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Control	20	0.04–3.22	1.22	0.848	8.74–49.23	19.62	10.171
Experimental	20	0.26–3.56	0.79	0.720	9.16–64.03	18.80	11.580

<sup>1</sup>Twenty larvae from each treatment were used, and each larva was tested three times. Statistics have been calculated for the mean values of time and distance per individual.



TABLE 2. Body size data for *Ambystoma maculatum* larvae raised with and without access to air

Character	Control (n = 20)			Experimental (n = 20)		
	Range	$\bar{x}$	SD	Range	$\bar{x}$	SD
Live snout-vent length (mm)	20.5–27.1	24.6	1.28	22.6–26.8	25.2	1.25
Live weight (g)	0.301–0.613	0.472	0.065	0.343–0.646	0.533	0.073
Preserved snout-vent length (mm)	19.5–24.4	22.6	1.11	20.7–25.1	23.1	1.19
Preserved lung surface area (mm <sup>2</sup> )	11.6–63.3	30.5	14.66	11.1–46.2	22.0	8.90

The body size and lung size data are summarized in Table 2. To evaluate the relationship between snout-vent length (SVL) and lung surface area (LSA), we used body size measurements taken after the larvae were fixed in formalin, inasmuch as the lung measurements were necessarily made after preservation. Because LSA should increase as a square of any linear dimension, we assumed a power relationship between the two variables, according to  $LSA = k \times SVL^2$ . Therefore, both variables were transformed to natural logarithms, and an analysis of covariance was performed on  $\ln LSA$ , with  $\ln SVL$  as covariate. Preliminary analysis showed no significant interaction between treatments and the covariate, which supports the assumption of homogeneity of slopes. The difference in  $\ln LSA$  between experimentals and controls, adjusted for the covariate, was significant ( $F = 6.550$ , 1, 37 d.f.,  $P = 0.015$ ), indicating smaller lungs in the larvae raised without access to air. In addition, a significantly higher proportion of these experimental animals had solidified lungs (experimentals: 12/20, controls: 5/20,  $G = 5.134$ , 1 d.f.,  $P < 0.025$ ). Standard paraffin histology revealed that this solidification was due to excess fibroblasts thickening the lung walls.

The difference in mean snout-vent length between treatments, using the values for living larvae, was nonsignificant ( $F = 2.096$ , 1, 38 d.f.,  $P = 0.156$ ). However, an analysis of covariance of  $\ln$  weight, taking  $\ln SVL$  as covariate, showed that the experimental larvae were heavier than the controls ( $F = 6.186$ , 1, 37 d.f.,  $P = 0.018$ ). The logarithmic transformation was required because weight varies as the cube of SVL. A direct evaluation of untransformed weights also yielded a significant difference between treatments ( $F = 7.630$ , 1, 38 d.f.,  $P = 0.009$ ). On average, when comparing animals of similar snout-vent length, deprivation of air during larval development in *A. maculatum* yielded an increase in mass of 13%, a reduction in lung surface area of 29%, and an increase of 35% in the

proportion of larvae that showed solidification of the lungs due to connective tissue thickening of their walls.

## DISCUSSION

Our experiment established a correlation among lung development, body mass, and the efficacy of the rheotactic response in the larvae of *Ambystoma maculatum*. Although we did not measure volumes, the difference in body mass, coupled with the absence of a difference in SVL, suggests that the specific gravity of experimental larvae was greater than that of controls. Thus we conclude that the experimental larvae were less buoyant than the controls, probably as a consequence of lung reduction/solidification. One inconsistency in the behavioral data was the lack of correlation between the distance and time variables. The difference between treatments in the former was not associated with a significant difference in the latter (although the difference between sample means was in the expected direction). Our interpretation is that experimental larvae descended more quickly to the streambed, where they were subjected to lower velocities. The controls, in contrast, tended to remain above the substratum in the higher velocities proportionately longer, and thus were carried more rapidly downstream before settling.

Collectively, our findings evaluate and support the hypothesis that lung reduction in amphibians has the selective advantage of reducing buoyancy. This adaptation is observed in several unrelated lineages of stream-dwelling salamanders (Wake, '66), as well as in tadpoles of stream-breeding frogs (Wassersug and Heyer, '88), and is especially important in the lungless plethodontid salamanders (Beachy and Bruce, '92).

Contrary hypotheses of a terrestrial rather than stream origin of plethodontid lunglessness are based on geologic history (Ruben and Boucot, '89; Ruben et al., '93). They have been formulated in response to a problematic center-of-origin model

for the time and place (late-Mesozoic Appalachia) of plethodontid origins (Wilder and Dunn, '20; Dunn, '26; Wake, '66). We suggest that it is more instructive to focus on the biology of living amphibians to resolve this question. For example, Booth and Feder ('91) have argued that physiological constraints associated with hypoxic boundary layers adjacent to the skin would counter selection for lung loss in salamanders in aquatic environments. However, there are highly aquatic plethodontids that remain submerged for extended periods as adults and breathe exclusively through the skin; such species have solved the boundary-layer problem by some unknown metabolic adaptation (Gatz and Piiper, '79; Booth and Feder, '91). In any case, the metabolic requirements of plethodontid salamanders are minimal in comparison with other small vertebrate ectotherms (Feder, '76, '83). Moreover, larval morphs have external gills in addition to the skin. Our finding that lung reduction and solidification are associated with an improved rheotactic response in larvae of an ambystomatid salamander reinforces the hypothesis that lung loss in plethodontids and lung reduction in other amphibians are adaptations to reduce buoyancy in lotic environments.

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