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Original Article

From Inflation to Flotation: Contribution of the Swimbladder to Whole-Body Density and Swimming Depth During Development of the Zebrafish (*Danio rerio*)

Benjamin W. Lindsey,[1,*] Frank M. Smith,[1,†] and Roger P. Croll[2,†]

Abstract

Teleost fishes have body tissues that are denser than water, causing them to sink. Many teleosts therefore possess a gas-filled swimbladder that provides lift, allowing fish to attain neutral buoyancy. The importance of the swimbladder as a buoyancy aid during changing body sizes over ontogeny and its role in determining the swimming depth of fish remain unclear. In this study, we have used the zebrafish (*Danio rerio*) to investigate changes in the size and shape of the swimbladder during development and examine whether these changes affect the hydrostatic contribution of the swimbladder during swimming. Our results showed that swim-up behavior is critical for larvae to first inflate their swimbladder, decrease body density, and attain neutral buoyancy. Following inflation, we found a strong linear correlation between fish volume and swimbladder volume over ontogeny. This trend was supported by measures of the density of zebrafish, which was conserved within a narrow range between 1.00 ± 0.001 and 0.996 ± 0.001 g/cm^3 despite an increase in the swimming depth of zebrafish, which occurred upon transition to a double-chambered organ. Finally, we demonstrated that the contribution of the swimbladder keeps the fish within 1.7% of neutral buoyancy throughout larval development.

Introduction

Teleost fish contain a variety of tissues, such as muscle, cartilage, and bone, which are denser than the external aqueous environment. Without compensatory mechanisms to provide buoyancy, fish would, therefore, sink. Consequently, the performance of essential behaviors such as feeding, predator avoidance, migrations, and reproduction[1] would be energetically costly as fish would need to swim constantly to maintain a vertical position in the water column. To overcome this problem, fish have evolved numerous mechanisms to achieve neutral buoyancy. These mechanisms include the development of watery muscle, synthesis of low-density lipids, and reduction in bone mass (reviewed in Refs.[1–3]). However, of all buoyancy compensating mechanisms, a gas-filled organ in the coelomic cavity, the swimbladder, is considered to be the most efficient.[2] By controlling the volume of gas in the swimbladder, fish can attain neutral buoyancy at any depth, thereby minimizing the amount of energy expended by swimming to hold vertical station in the water column.

To date, most studies on teleost swimbladders have focused either on the initial inflation of the larval organ[4–12] or on the form and function of the adult swimbladder.[3,13–27] Little attention has been paid to the hydrostatic contribution of the swimbladder during development. In particular, information is lacking on how developmental changes in swimbladder volume may affect the ability of fish to maintain neutral buoyancy and how these changes in swimbladder morphology impinge on ontological changes in behaviors.

In this study, we employ the zebrafish as a model for examining swimbladder development. This species possesses well-known advantages in terms of rapid development to maturity and transparency of the larval body wall,[28] permitting visualization of early swimbladder growth *in situ*. Moreover, numerous genes that appear to be involved in swimbladder development have now been identified.[29,30] In addition, the anatomy of the adult swimbladder has been described with respect to its musculature, vasculature, and innervation,[31] as well as the contribution of the swimbladder to the whole-body density of the adult fish.[32] Finally, a microscopic study of the development of the swimbladder has

Departments of [1]Anatomy and Neurobiology and [2]Physiology and Biophysics, Dalhousie University, Halifax, Canada.
*Present address: Department of Cell and Systems Biology, University of Toronto, Toronto, Canada.
†These two authors contributed equally to this work.

recently appeared detailing the ontological changes in the ultrastructure of the organ wall, along with the development of its musculature, vasculature, and innervation.[33] In this study, we examine how changes in the size and shape of the swimbladder contribute to the attainment of neutral buoyancy over the course of ontogeny. Lastly, we investigate how such changes in the swimbladder correlate with alterations in swimming depth during development. A preliminary version of this report has appeared elsewhere.[34]

Materials and Methods

Animals

Adult zebrafish (*Danio rerio*, Hamilton-Buchanan) of both sexes were purchased from a local pet store (Aqua Creations Tropical Fish, Halifax, Canada) and kept in tanks with aerated, dechlorinated tap water at 26°C–28°C on a 14:10-h light (from 08:00) and dark (from 22:00) cycle. Fish were maintained and bred following procedures outlined by Westerfield.[35] Eggs and larvae were reared until zebrafish reached the appropriate stage of swimbladder development. All zebrafish were sacrificed by an overdose of 0.4% tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma Chemical, Mississauga, Canada) for at least 1 min or until respiration had ceased. All protocols followed the *Guide to the Care and Use of Laboratory Animals* as established by the Canadian Council for Animal Care and institutional approval was obtained from the University Committee on Laboratory Animals at Dalhousie University.

Morphometric experiments

Total body length, density, mass, and volume, as well as swimbladder volume, were measured to determine the contribution of the swimbladder to whole-body density at all stages of zebrafish development: preinflation (PI) ($n = 46$), early single-chamber (ESC) ($n = 75$), late single-chamber (LSC) ($n = 45$), early double-chamber (EDC) ($n = 29$), late double-chamber (LDC) ($n = 39$), and adult ($n = 25$). The total body length of zebrafish was measured from the tip of the lower jaw to the end of the caudal fin.[33] Procedures for measuring fish density, mass, and volume varied at different stages of swimbladder development and are discussed below. Correlations between fish volume and mass ($n = 259$), and swimbladder volume and fish volume ($n = 213$) were also investigated over the ontogeny of zebrafish.

Larvae at PI-LDC stages of swimbladder development. Images of the left lateral aspect of inflated swimbladders were taken through the transparent body wall (ESC, LSC) or following excision of the organ (EDC, LDC), for subsequent volume calculations. In EDC and LDC stage larvae, whole-body density was estimated before dissection. The mass of individual larvae from PI-EDC stages was measured by taring the total mass of the cap of a 0.5-mL Eppendorf tube, a drop of water, and a 0.5-cm^2 piece of Parafilm on an electronic scale (resolution $\pm$ 0.001 g). Thereafter, larvae were blotted to remove surface water and placed into the drop, the cap was sealed with Parafilm to prevent evaporation, and the mass was recorded. The mass of larvae at the LDC stage was measured directly on the scale following blotting. Next, larvae at PI-LSC stages were immediately transferred into an aqueous sucrose mixture with a vertical density gradient. This method has been commonly employed to obtain the density of small tadpoles[36] and teleost larvae.[37,38] A total of 14 sucrose solutions ranging in density from 1.005 to 1.074 g/cm^3, at a constant temperature of 28°C, were made based on the equation of Barber.[39] The separate sucrose solutions were then layered in 20 mL volume intervals in a 300-mL graduated cylinder beginning with the solution of greatest density and ending with freshwater. Alternatively, older (EDC and LDC) larvae were placed sequentially into separate sucrose solutions of increasing density to estimate the density of larvae. The density of the solution in which larvae came to rest in the vertical gradient (PI-LSC) or the density of the first separate test solution in which larvae remained neutrally suspended (EDC-LDC) for a maximum of 2 min was taken as the density of the specimen.

Finally, as PI-LDC stage animals were too small to obtain accurate direct measures of total body volume by water displacement, we calculated the value using mass and density measurements, as described earlier.

Adult zebrafish. Measurements were made on zebrafish of both sexes (males = 12; females = 13). Length, mass, volume, and density were calculated according to the procedure of Robertson *et al*.[32] In brief, total body length was measured, fish were blotted with gauze to remove surface water, and the mass was determined on an electronic scale (resolution $\pm$ 10 mg). A volumetric chamber, described in detail by Robertson *et al*.,[32] was used to measure fish volume by water displacement. The density of adult fish was calculated as the ratio of mass to volume. No significant difference (independent samples *t*-test, $p = 0.463$) was observed between the density of adult male and female zebrafish in our study, as was consistent with previous findings of Robertson *et al*.,[32] who reported that whole-body density did not differ between male, gravid female, and nongravid female zebrafish. All samples of adult fish were therefore pooled for subsequent comparisons. Thereafter, the intact swimbladder was excised from the coelomic cavity through a ventral incision and an image of the left lateral aspect of the swimbladder was taken for volume calculations.

Swimbladder volume. The volume of the swimbladder was estimated stereologically for all zebrafish. The accuracy of this method compared with direct measurement by puncture and collection of gas from the swimbladder is supported by earlier studies in adult zebrafish[32] and yellowfin tuna.[40] All geometrical formulas employed were from Beyer.[41] Volume estimations were calculated from photographs of the lateral aspect of single- and double-chambered swimbladders.

Representative overlays of the geometrical shapes used to calculate swimbladder volume, along with their linear dimensions and corresponding equations (V1–V5) are shown in Figure 1 and Table 1, respectively. The volume of single-chambered swimbladders (ESC, LSC) or the anterior chamber of double-chambered swimbladders (EDC, LDC, adult) was modeled as a rotary prolate ellipsoid (V1, Fig. 1A–C). This geometrical figure has been confirmed to provide a reliable approximation of volumes of spheroid- or ovoid-shaped organs and has been used previously for estimations of single-chambered swimbladders.[4,38,42–44] The volume of the posterior chamber of zebrafish at the EDC, LDC, and adult stages

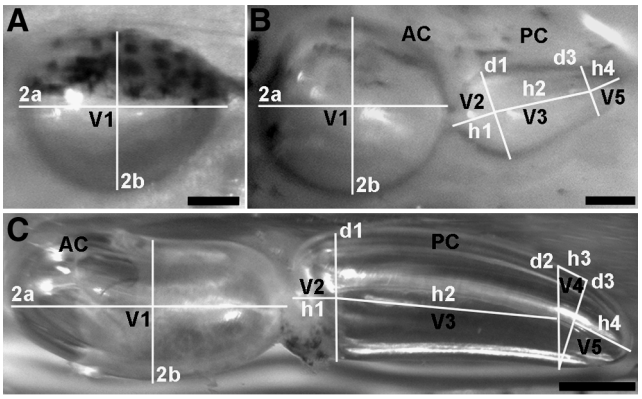

FIG. 1. Photomicrographs of lateral views of representative swimbladders (anterior is left, dorsal is up) illustrating the overlay and linear dimensions of geometric shapes used to estimate swimbladder volume as explained in Table 1. (**A**) Single-chambered swimbladder approximated by a rotary prolate ellipsoid (V1). (**B**) LDC and (**C**) adult swimbladders showing the anterior (AC) chamber approximated by VI and the posterior chamber (PC) approximated by the sum of the combination of volumes V2–V5. For an explanation of equations see Table 1. Scale bars: (**A**, **B**) 0.2 mm; (**C**) 0.5 mm.

was estimated by a combination of up to four geometrical shapes (V2–V5, Fig. 1B, C). For double-chambered swimbladders, the sum of all constituent geometrical units was taken as the total swimbladder volume. Following calculations of swimbladder volume, the ratio of swimbladder volume to fish volume for zebrafish at each stage of swimbladder development was calculated. The ratio of the anterior to posterior chamber volume for all double-chambered swimbladders was also calculated.

Finally, the contribution of the swimbladder to whole-body density at all stages of swimbladder development was determined for individual animals. This was done by comparing the whole-body density of fish as calculated above to that of the same fish after the swimbladder had been mathematically subtracted from the same fish volume. The mass of gas in the swimbladder was assumed to be negligible.

Behavioral studies

The depth of groups of zebrafish or individuals within aquarium tanks was recorded with a CCD video camera (Honeywell Model HCM574E, Syosset, NY) aimed at the front panel of each tank. The video camera was connected to a computerized digital recording system (Astra 8 Video Surveillance System; Pace Setter Technologies, Dartmouth, Canada). One-hour segments were recorded every second hour from 09:00 to 20:00 h. The depths of fish were scored at 1 cm intervals with the aid of a scale placed on the tank front panel.

Swim-up behavior. Zebrafish larvae ($n = 47$) were observed from 2 days postfertilization (dpf) until they were free-swimming in the water column, to investigate larval swim-up behavior. Here, swim-up behavior was defined as the behavior of larvae from hatching to when they reached the surface for initial inflation of the swimbladder; this period overlapped the PI stage of swimbladder development. Both the depth of larvae during swim-up behavior and the movement of larvae toward the surface of the water were examined.

Ten fertilized eggs from each of five trials were placed into a 1-L observation tank, which was 9 cm deep. Water temperature was standardized to 28°C, because even slight deviations in temperature are known to affect the time of hatching and duration of swim-up behavior.[5,7] Eggs and larvae were video recorded daily during daylight hours until larvae became free-swimming in the water column. The depth of larvae was noted in single frames selected at 30 min intervals. The mean depth of larvae was calculated for each day of recording, with separate days treated as repeated measurements for analysis.

Substrate adhesion. A combination of video recordings and direct observations was used to monitor the ability of larvae from 2 to 4 dpf to use different substrates to reach the surface of the water for swimbladder inflation. Substrates were chosen based on field reports on the natural environment of zebrafish.[45,46] In this study, the three substrates chosen were small rocks, coarse sand, and aquatic vegetation (*Vallisneria americana*, Mini Twister). Rock and sand substrates were created by using sheet-plastic tank inserts onto which a layer of sand was glued using a nontoxic spray adhesive or onto which rocks were fastened using modeling clay. These inserts were positioned vertically along one of the inside walls of a 9-cm-deep tank. Vegetation was anchored in the center of the tank using rocks, with a small number of leaves emerging from the surface of the water to allow larvae access

Table 1. Geometrical Figures and Equations Used to Estimate the Total Swimbladder Volume of Zebrafish

Volume	*Geometrical figure*	*Equation*	*Details*
V1	Rotary prolate ellipsoid	$V1 = 4/3\pi ab^2$	$a = 0.5$, major axis; $b = 0.5$, minor axis
V2	Zone and segment of one base of a sphere	$V2 = 1/6\pi h1\ (3r_1{}^2 + h1^2)$	$r_1 = 0.5d1$, measured at the widest point of the rostral portion of the posterior chamber
V3	Frustum of a cone	$V3 = 1/3\pi h2\ (r_1{}^2 + r_2{}^2 + r_1r_2)$	$r_1 = 0.5d1$ $r_2 = 0.5d2$ $d2$ taken as 0.9 $d1$ because of the ventral curvature of the swimbladder
V4	Half cylinder	$V4 = 1/2\pi r_2{}^2h3$	
V5	Cone	$V5 = 1/3\pi r_3{}^2h4$	$d3$ taken as the base of the cone $r_3 = 0.5d3$

to the air–water interface. During trials, up to 20 hatchlings at a time were placed in tanks including one of the three substrates or in tanks without added substrate as a control. Larvae were deemed capable of attaching to a vertical substrate if they had adhered for a minimum of 2 min.

Swimming depth during development. Zebrafish were observed individually in the water column at ESC ($n = 16$), LSC ($n = 15$), EDC ($n = 10$), LDC ($n = 12$), and adult ($n = 12$) stages of swimbladder development to examine changes in swimming depth. The water depth and tank volume varied by stage because of the range of fish sizes observed during this study (9 cm depth/1 L volume, ESC, LSC; 16 cm/4.9 L, EDC; 36 cm/22.5 L, LDC; 36 cm/64.8 L, adult). For each trial, zebrafish were provided an overnight acclimation period from 20:00 to 09:00 h. The next morning, fish were video recorded beginning at 09:00 h, and depth was observed in single frames every 6 min within each 1-h recording segment. The mean depth of fish was calculated separately for each recording hour and treated as repeated measurements for analysis. However, our data showed no significant effect of time of day on mean depth of zebrafish during the six 1-h recording segments (ESC-adult: one-way repeated measures analysis of variance [ANOVA], $p > 0.05$) for all stages of swimbladder development following inflation. Thus, the depth of zebrafish at each stage was pooled across all 6 h recorded, to obtain a mean depth representative of the entire daylight recording period. A pooled mean depth was also calculated for larvae at the PI stage ($n = 47$) using data from days 2 to 4 of swim-up behavior to extend comparisons across all stages of swimbladder development.

Statistical analysis

Values are expressed as mean ± standard error. The significance level for all experiments was set at $p \leq 0.05$. Pairs of means were compared using independent samples *t*-tests, whereas multiple means comparisons were performed using a one-way ANOVA or a one-way repeated measures ANOVA. When multiple means comparisons showed a significant difference, individual means were compared using a Dunnett's T3 *post hoc* test for unequal variances. Linear regression was used to examine correlations between morphometric measurements. Statistical calculations were done using SPSS 14.0 (SPSS, Chicago, IL).

Results

Stages of swimbladder development

Representative images of each stage of swimbladder development are shown in Figure 2. Detailed descriptions of these stages have been reported previously.[33] A brief summary of each stage is given below with additional qualitative descriptions.

Preinflation. The PI stage extended from hatching to when larvae had reached a total body length of approximately 3.4 mm (Fig. 2A). At this stage the uninflated swimbladder had evaginated from the esophageal wall but remained attached by a nascent pneumatic duct. A prominent yolk sac was visible at this time and was located ventral to the developing digestive tract.

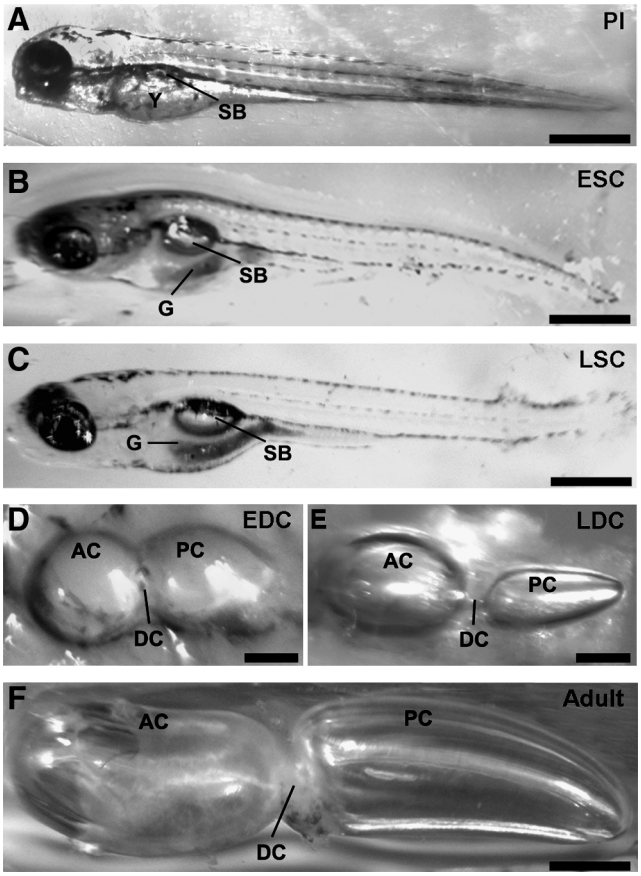

FIG. 2. Photomicrographs of the stages of swimbladder development as viewed through the transparent body wall of larvae (**A–C**) or following excision from the coelomic cavity (**D–F**). All images show left lateral view. (**A**) Preinflation (PI) stage larva showing the location of the uninflated swimbladder (SB) in relation to the yolk sac (Y). (**B**) Early single-chamber (ESC) stage larva showing the inflated swimbladder adjacent to the developing gut (G). (**C**) Late single-chamber (LSC) stage larva showing the ovoid shape of the swimbladder in the coelomic cavity. (**D**) Early double-chamber (EDC) stage swimbladder displaying the anterior (AC) and posterior (PC) chambers connected by a wide ductus communicans (DC). (**E**) Late double-chamber (LDC) stage swimbladder showing the enlarged anterior chamber and constriction of the ductus communicans. (**F**) Adult swimbladder showing the ellipsoid-shaped anterior chamber and elongated posterior chamber. Scale bars: (**A–C**, **F**) 0.5 mm; (**D**, **E**) 0.2 mm.

Early single-chamber. The beginning of the ESC stage was marked by first inflation of the swimbladder when larvae were approximately 3.5 mm in body length; this stage extended to when larvae had a total body length of about 4.4 mm (Fig. 2B). The swimbladder was roughly spherical within the coelomic cavity and lay dorsal to the esophagus and ventral to the notochord.

Late single-chamber. By the LSC stage the swimbladder formed a more elongated, ovoid shape (Fig. 2C), as larvae grew from 4.5 to 5.9 mm total body length.

Early double-chamber. Larvae at the EDC stage had total body lengths ranging from 6.0 to 9.4 mm (Fig. 2D). This stage was distinguished by the formation of the second (anterior) chamber from the cranial aspect of the original (now posterior) chamber. The anterior chamber formed as a spherical or slightly ovoid extension and appeared to inflate immediately upon evagination. After inflation the anterior chamber generally appeared to be slightly larger than the posterior chamber and separated from the latter only by a partial restriction, which later deepened to form the "ductus communicans."

Late double-chamber. The beginning of the LDC stage was characterized by the narrowing of the ductus communicans connecting the anterior and posterior chambers (Fig. 2E). Larvae at this stage grew from 9.5 mm to approximately 20 mm total body length, although it was not uncommon for larvae at the LDC stage to measure up to 25 mm. Both chambers elongated along the longitudinal axis during this stage to approach the adult form. However, at this stage the anterior chamber typically appeared to be much larger than the posterior chamber.

Adult swimbladder. The swimbladder took on the adult form when zebrafish reached a total body length between 20 and 25 mm (Fig. 2F). At this point, the anterior chamber was generally ellipsoid, while the posterior chamber was more elongated and narrowed at its caudal-most aspect. Although of different shapes, the two chambers appeared to have roughly equal volumes at this stage.

Swimbladder volume during development

There was a strong relationship during development from hatching to adulthood between fish volume and mass (Fig. 3A; linear regression, $r^2 = 0.99$, $p < 0.001$), suggesting the need for a progressively larger swimbladder volume to maintain neutral buoyancy as development proceeded. By calculating the total swimbladder volume of zebrafish at each stage of development, we found that swimbladder volume and fish volume were also correlated over ontogeny (Fig. 3B; linear regression, $r^2 = 0.93$, $p < 0.001$). The total mean swimbladder volume (Table 2; one-way ANOVA, $p < 0.001$) and the separate volumes of the anterior and posterior chambers (Table 2; one-way ANOVA, $p < 0.001$) increased significantly during development. The volume of the anterior chamber was significantly greater than the posterior chamber at the EDC and LDC stages (Table 2). The corresponding mean ratios of the anterior to posterior chamber volume were calculated to be 2.03 ± 0.35 (EDC), 4.50 ± 0.49 (LDC), and 1.15 ± 0.07 (adult).

The ratio of swimbladder volume to fish volume has been commonly used across fish species to assess the contribution of the swimbladder to whole-animal buoyancy.[44,47] Our results showed significant variation in this relationship across stages of development in the zebrafish (Table 2; one-way ANOVA, $p < 0.05$).

Whole-body density during development

Whole-body density of intact zebrafish was measured and then recalculated after subtracting the volume of the swimbladder from that of the entire fish. The density of whole larvae decreased significantly from 1.04 ± 0.001 to 0.996 ± 0.001 g/cm^3 upon initial inflation of the swimbladder at the ESC stage (Fig. 4; independent samples t-test, $p < 0.001$). Thereafter, the body density of animals with inflated swimbladders remained within a narrow range from 0.979 ± 0.007 to 1.00 ± 0.001 g/cm^3 across all stages of swimbladder development (Fig. 4). Thus, our data indicate that the volume of the swimbladder contributes to the maintenance of whole-body density of these fish within 1.7% of the density of fresh water at 28°C (0.996 g/cm^3). Following mathematical subtraction of swimbladder volume, mean density of larvae was significantly greater (Fig. 4; paired-samples t-test, ESC-adult: $p < 0.001$) than the density of animals with swimbladders from the ESC stage onward, demonstrating that this organ was effective at significantly reducing body density.

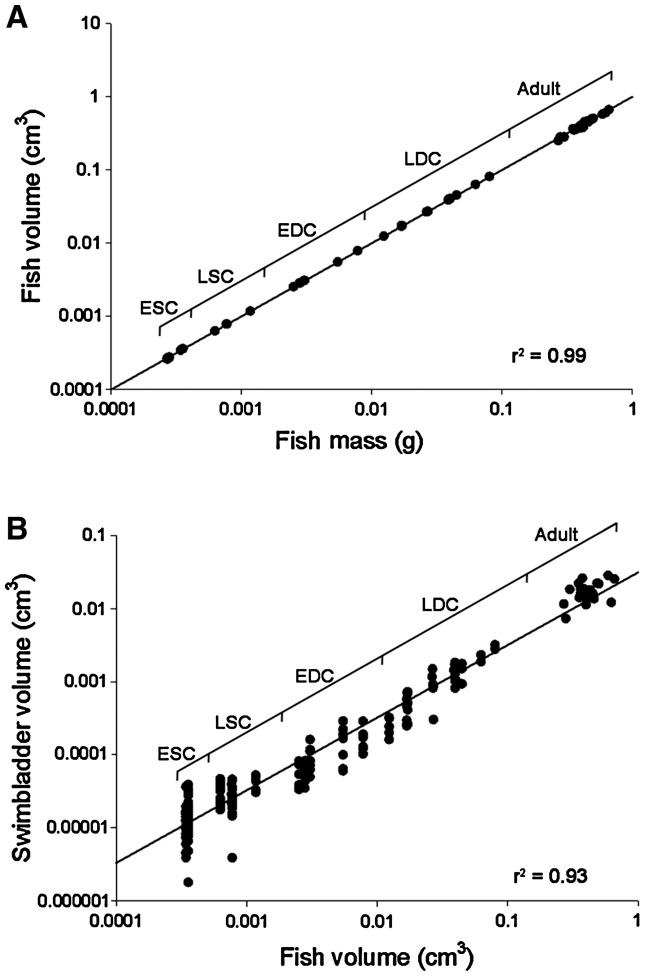

FIG. 3. Log–log plots of the relationships among fish and swimbladder volume and mass over the course of swimbladder development. (**A**) Co-relationship between fish volume and mass across all stages of swimbladder development ($r^2 = 0.99$, $n = 259$). (**B**) Co-relationship between swimbladder and fish volume post swimbladder inflation ($r^2 = 0.93$, $n = 213$). The approximate ranges of fish masses and volumes at the ESC, LSC, EDC, LDC, and adult stages are indicated.

Table 2. Mean Morphometric Measurements Made on Whole Animals and Inflated Swimbladders

Stage (body length range in mm)	*Total body length (mm)*	*Mass (g)*	*Fish volume (cm^3)*	*Swimbladder volume (cm^3)*			*SB:fish volume (%)*
				Total	*Anterior*	*Posterior*	
PI (3.0–3.4)	3.2 ± 1.3	$2.8\times10^{-4} \pm 0.26$	$2.6\times10^{-4} \pm 0.29$	–	–	–	–
ESC (3.5–4.4)	3.8 ± 0.8	$3.5\times10^{-4} \pm 0.23$	$3.5\times10^{-4} \pm 0.23$	$1.5\times10^{-5} \pm 6.0$	–	–	4.2 ± 6.0
LSC (4.5–5.9)	5.0 ± 1.4	$9.6\times10^{-4} \pm 9.0$	$9.5\times10^{-4} \pm 9.1$	$3.2\times10^{-5} \pm 6.9$	–	–	3.6 ± 5.8
EDC (6.0–9.4)	7.4 ± 2.6	$4.4\times10^{-3} \pm 8.0$	$4.4\times10^{-3} \pm 8.2$	$1.1\times10^{-4} \pm 11.8$	$7.2\times10^{-5} \pm 16.7^{a}$	$4.1\times10^{-5} \pm 10$	$2.6 \pm 8.1^{b,c}$
LDC (9.5–20.0)	13.6 ± 3.3	$3.0\times10^{-2} \pm 9.7$	$3.0\times10^{-2} \pm 9.7$	$9.6\times10^{-4} \pm 12.5$	$7.4\times10^{-4} \pm 12.2^{a}$	$2.2\times10^{-4} \pm 15.9$	2.9 ± 5.9^{b}
Adult (20.1–40.0)	35.8 ± 1.2	$4.1\times10^{-1} \pm 4.9$	$4.2\times10^{-1} \pm 4.5$	$1.8\times10^{-2} \pm 5.6$	$9.4\times10^{-3} \pm 7.7$	$8.2\times10^{-3} \pm 4.9$	$4.3 \pm 5.3^{d,e}$

Values are expressed as mean ± % error of mean. A significant increase in the total, anterior chamber, and posterior chamber swimbladder volume was noted at each successive stage of development (Dunnett T3, $p < 0.001$); therefore, no notation is shown.

[a]A significant difference between the volume of the anterior and posterior swimbladder chambers at the EDC and LDC stages (Dunnett T3, $p < 0.001$).

Significant differences in SB:fish volume among stages of swimbladder development are denoted as follows: [b]different from ESC, [c]different from LSC, [d]different from EDC (independent samples *t*-test, $p = 0.019$), [e]different from LDC (independent samples *t*-test, $p < 0.001$).

SB, swimbladder; PI, preinflation; EDC, early double-chamber; ESC, early single-chamber; LSC, late single-chamber; LDC, late double-chamber.

Swim-up behavior

Five groups of 10 fertilized eggs were observed from hatching until larvae were free-swimming in the water column to assess the duration and method used to reach the surface of the water during swim-up behavior to inflate the swimbladder (Figs. 5 and 6). Eggs hatched at 2 dpf, at which time all larvae were located along the bottom of the 9.0-cm-deep observation tank (mean observed depth of larvae = 8.9 ± 0.1 cm) and displayed little movement. Within hours after hatching, larvae began active, randomly directed movements along the tank bottom until they came into contact with a vertical surface, at which point they attached to the surface approximately 0.5–1.0 cm above the tank bottom (Fig. 5A, 2 dpf; Fig. 5B, steps 1–2). Larvae adhered to the tank walls using the rostro-ventral surface of their body and oriented head uppermost, with the body aligned nearly vertically (Fig. 6A). This form of surface attachment was also observed during experiments involving separate groups of larvae with access to surfaces that were more natural than the aquarium sides, including rock (Fig. 6B, B′; $n = 7$), sand (Fig. 6C, C′; $n = 6$), and aquatic vegetation (*Vallisneria americana*); the latter was the only substrate to which larvae did not attach in our study.

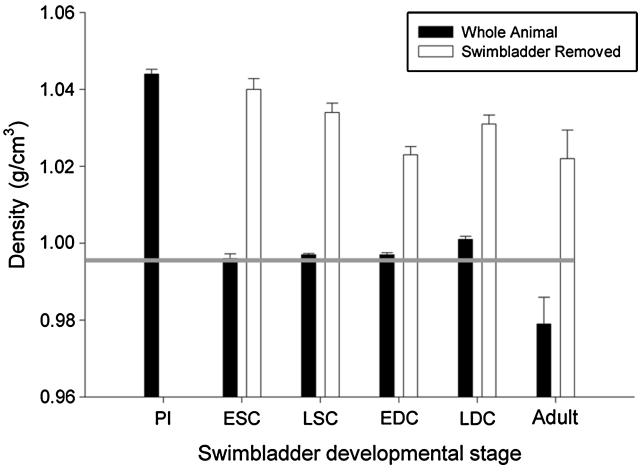

FIG. 4. Whole-animal density (black bars) and the density of larvae with the swimbladder density contribution subtracted (white bars) at each stage of swimbladder development. Upon swimbladder inflation, the density of larvae at the ESC stage significantly decreased from PI stage and was brought close to neutral buoyancy (density of water calculated as 0.996 g/cm^3, gray line) at 28°C, the temperature of the tank water in this study. Comparisons of the density of fish before and after mathematical subtraction of the swimbladder at each stage of development showed that the density significantly increased in all cases. Therefore, no notation of significance is marked on the graph for this comparison. PI, $n = 46$; ESC, $n = 75$; LSC, $n = 45$; EDC, $n = 29$; LDC, $n = 39$; Adult, $n = 25$.

Examination of a subset of larvae ($n = 15$) showed considerable variation in the length of time larvae remained attached at one position on the tank wall, ranging from 1 to 240 min. This was typically followed by larvae releasing their holds from the tank wall and propelling themselves upward using a short series of tail flicks, before reattaching at a shallower depth on the same surface (Fig. 5B; step 3). The distance moved by a single larva between periods of attachment generally spanned 0.5–4.0 cm. This behavior was used by larvae during the PI stage from 2 to 4 dpf to reside at significantly shallower depths along the tank walls (Fig. 5A; one-way repeated measures ANOVA, $p = 0.001$) and was most pronounced between 3 and 4 dpf when 74.5% and 100% of larvae, respectively, moved closer to the surface of the water. The sequence of detachment, tail flicking, and reattachment was repeated until larvae were able to break the air–water interface and begin to swallow air bubbles to inflate their swimbladders. After this event the animals were free-swimming just below the surface of the water (Fig. 5B; step 4). At the beginning of 4 dpf, only 14.9% of larvae had inflated their swimbladders and were already free-swimming (at a mean depth of 1.9 ± 0.1 cm), whereas the majority of larvae were continuing to ascend the tank walls to the surface of the water (at a mean depth of 4.0 ± 0.9 cm) (Fig. 5A). By the end of 4 dpf, all zebrafish had reached the surface of the water and were free-swimming.

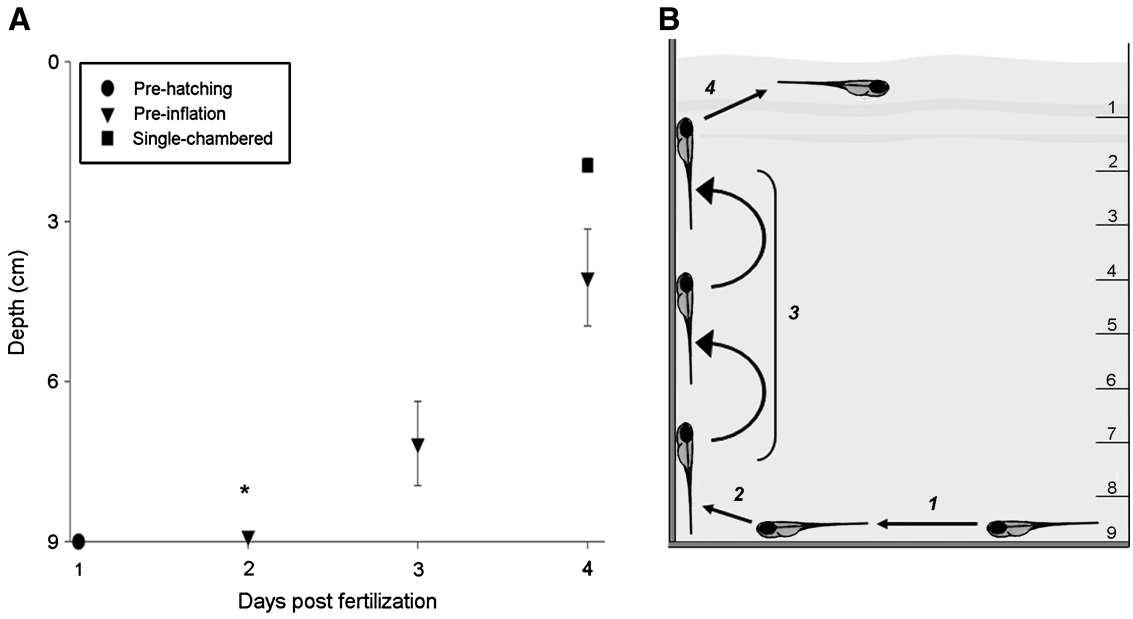

FIG. 5. Swim-up behavior of zebrafish larvae ($n = 47$). (**A**) Larval depth was observed over four 6-h video-recording sessions during daylight period beginning at fertilization. Swim-up behavior began following hatching at 2 dpf, coinciding with the onset of the PI stage. At 2 dpf, only 14.9% of larvae actively began to ascend the tank walls toward the air–water interface, whereas 74.5% displayed this behavior by 3 dpf. By 4 dpf, 85.1% of larvae continued to ascend the tank walls toward the surface of the water (triangle), with the remainder already free-swimming in the water column at early-single chamber stage (ESC, square). An asterisk (*) denotes a significant difference between the depth of larvae at the PI stage from days 2 to 4. (**B**) Schematic diagram showing the stereotypical movements of larvae during swim-up behavior. (1) Larvae move along the bottom until making contact with the tank wall. (2) Larvae first adhere to the wall at 0.5–1.0 cm above the tank floor. (3) Larvae repeatedly release from the tank wall, perform tail flicks to propel themselves upward, and reattach at a shallower depth, approaching the air–water interface. (4) Larvae break the air–water interface and take in air bubbles to inflate swimbladder; this results in neutral buoyancy with larvae positioned just below the surface of the water. Scale on both panels represents the water depth in centimeters.

Swimming depth during development

The overall trend in the depth of zebrafish in the water column from PI to the adult stage is shown in Figure 7. Because of the differences in tank depth used in this study, statistical comparisons were performed only among groups of larvae in observation tanks with similar depths. Before swimbladder inflation, larvae at the PI stage had a mean depth of 6.7 ± 0.7 cm as they ascended the tank walls toward the surface of the water. Observed depths of larvae differed significantly over ontogeny (one-way ANOVA, $p < 0.001$). Moreover, pairwise analyses revealed a significant difference between the depth of larvae at the PI stage and the ESC and LSC stages (Dunnett T3, $p < 0.001$). However, no significant difference was observed between the depth of larvae at the ESC (1.4 ± 0.1 cm) and LSC stages (1.9 ± 0.2 cm; Dunnett T3, $p = 0.227$). The transition of the swimbladder from a single- to a double-chambered organ correlated with the onset of increased depth in the water column. This was first observed at the EDC stage (depth 5.1 ± 0.9 cm), followed by an increase in the depth of larvae at the LDC stage to approximately the middle of the 36-cm-deep observation tank (17.6 ± 1.2 cm). The depths of zebrafish at the LDC and adult stages (13.4 ± 1.3 cm) were also significantly different from each other (independent samples *t*-test, $p = 0.031$).

Discussion

This study describes the contribution of the swimbladder to the maintenance of neutral buoyancy over the course of zebrafish development. We showed that before swimbladder inflation, larval whole-body density was significantly greater than that of the surrounding aquatic medium. Young larvae must actively move upward in the water column to the surface during the first 2–4 dpf to inflate the swimbladder to attain near neutral buoyancy. We found that zebrafish maintained a density close to that needed for neutral buoyancy in fresh water at all stages of development subsequent to swimbladder inflation. Moreover, our behavioral data demonstrated that the mean swimming depth of zebrafish in the water column over the daylight photoperiod may in part be correlated with stages of swimbladder development.

Swim-up behavior

Initial swimbladder inflation is a critical stage of development for physostomous fishes, such as the zebrafish, which maintain a connection between the esophagus and the swimbladder into adulthood.[5,11,12,37] The behavior associated with the initial inflation of the swimbladder, generally referred to as "swim-up behavior,"[48–51] is one of the first coordinated behaviors displayed by larvae following hatching (reviewed in Ref.[52]). Inability to inflate the swimbladder has been linked with developmental abnormalities,[9] as well as an increase in metabolic rate and oxygen consumption.[6] In many cases, there is a narrow time window during which swimbladder inflation is optimal; after this period the chance of inflation is significantly reduced.[5,8] Timely inflation is

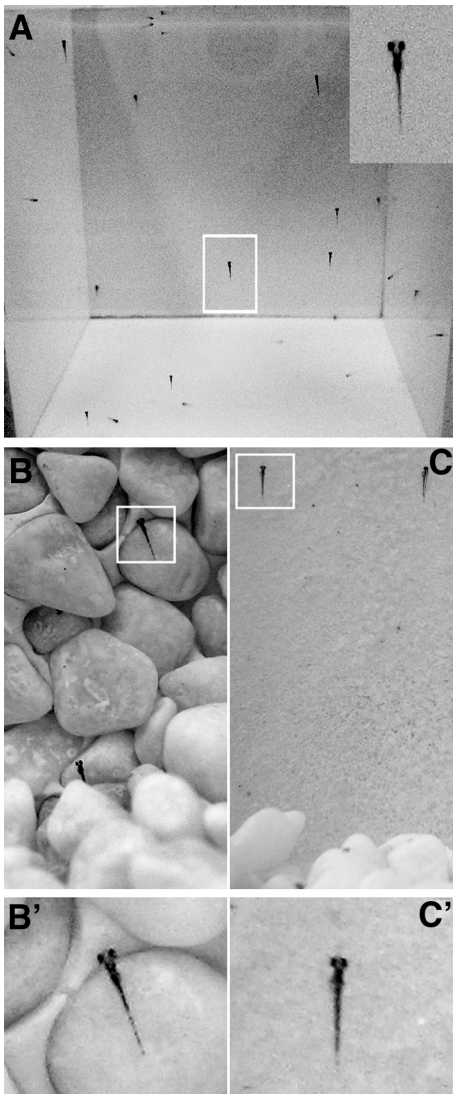

FIG. 6. Images of larval zebrafish during swim-up behavior (**A**) and substrate adhesion (**B**, **C**). (**A**) Front view of a 9-cm-deep tank showing the vertical orientation of larvae along control tank walls during swim-up. Inset shows a typical larva with head oriented upward. (**B**, **C**) Zebrafish larvae attached to vertical rock (**B**, inset **B′**, $n = 7$) and sand (**C**, inset **C′**, $n = 6$) substrates at the PI stage. On both experimental substrates, larvae adhered in the same manner as in the control tank (**A**), with their heads oriented upward. Regions enclosed in boxes in **B** and **C** depict the location of insets in **B′** and **C′**.

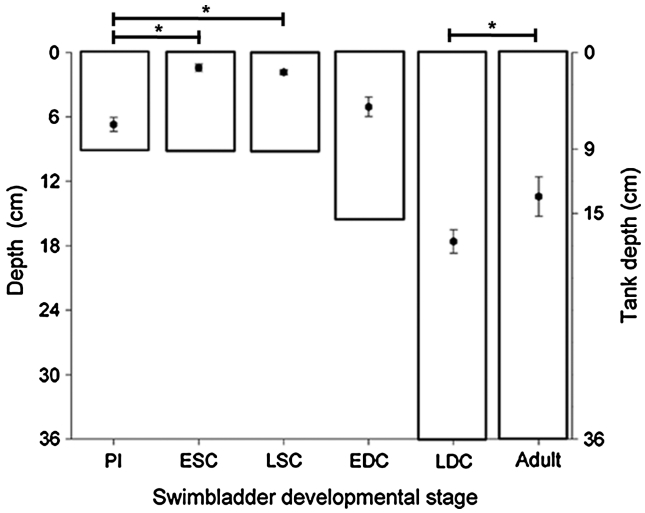

FIG. 7. Mean depth of zebrafish at each stage of swimbladder development. Before swimbladder inflation, PI stage larvae ($n = 47$) were attached along the tank floor and walls. Larvae at the ESC ($n = 16$) and LSC ($n = 15$) stages were swimming just below the surface. The formation of the second chamber (anterior) at the EDC stage ($n = 10$) was correlated with larvae beginning to increase swimming depth. The greatest shift in depth occurred as larvae entered the LDC stage ($n = 12$) when fish were located at mid-water depth. Adult zebrafish were observed at a mean depth ($n = 12$) similar to that of larvae in the LDC stage. Rectangles denote the depth of tanks used to evaluate the depth of zebrafish at each stage of swimbladder development.
*, A significant difference from the depth of larvae at the PI stage and a significant difference from the depth of zebrafish between LDC stage and adulthood were observed.

particularly important for cyprinids, such as zebrafish, which use the swimbladder as an organ both for hydrostatic control and, in conjunction with the Weberian ossicles, for audition.[15,53,54]

Despite the biological importance of swim-up behavior in the development of swimbladder-bearing teleosts, few studies have described how larvae reach the surface of the water to first inflate the swimbladder. We have shown that larvae hatch at 2 dpf in 28°C water (Fig. 5A), consistent with earlier reports by Kimmel *et al.*[55] Thereafter, swim-up behavior is completed within a 2-day period in 9-cm-deep tanks under laboratory conditions. Upon hatching, larvae at the PI stage have a density significantly greater than that of the surrounding water (Fig. 4). Thus, movement toward the air–water interface can only be accomplished by upward movements comprised of spontaneous tail flicking (Fig. 5B) with interim adherence to vertical surfaces (Fig. 6A). One earlier study proposed that larval attachment during swim-up behavior in zebrafish is carried out using secretory cells located in the epidermis of the head, under the eye and nasal depression, around the mouth, and toward the postoral region[56] (also reviewed in Ref.[57]).

In the wild, the substrates of the stream habitats of zebrafish typically include clay, silt, stone, and aquatic plants.[45,46] We showed that larvae attach to vertical rock and sand surfaces without difficulty (Fig. 6B, C), but not to the aquatic vegetation used in this study, although attachment to other forms of aquatic vegetation has been previously documented.[56] Thus, zebrafish larvae in the wild likely attach to a combination of substrates of different textures to reach the air–water interface. Our results suggest that net upward movement of larvae is slow during swim-up, raising the question of whether swim-up behavior from greater depths would be prolonged beyond 4 dpf. To date it is also unclear whether there exists a limiting depth from which larvae can ascend to inflate the swimbladder, although female zebrafish in the wild may avoid this problem by laying eggs only in shallow water.

Buoyancy of zebrafish with single-chambered swimbladders

Upon inflation of the single-chambered swimbladder, the density of zebrafish larvae decreased significantly (Fig. 4), bringing the buoyancy of larvae close to neutral buoyancy. This is consistent with the magnitude of reduction of whole-fish densities reported for other teleosts upon inflation of the swimbladder.[4,37,38,58–60] Further, the timing of swimbladder inflation reported here is in agreement with earlier reports on inflation in the developing zebrafish at approximately 4 dpf.[30,33]

Only one other study has examined the relationship between swimbladder inflation and volume in larval zebrafish. Goolish and Okutake[9] calculated the gas volume relative to body size (V_{RG}) of larval zebrafish swimbladders. Applying these authors' analytical technique to data from this study at the corresponding LSC stage, we calculated a considerably lower mean V_{RG} compared with their value. The reason for this discrepancy is unclear but two explanations may account for this difference. First, the zebrafish examined in the two studies came from different stock populations, which appeared to have had slightly different growth rates, resulting not only in different body lengths but possibly other dimensions as well. Second, different diets were used in these studies during early development of the larvae and may have resulted in different body compositions (e.g., lipid contents), which may have affected tissue densities. Taken together, these differences in swimbladder volume during the early development of zebrafish highlight the difficulty in making concrete comparisons even within the same species, given that factors such as water temperature, rearing conditions, and feeding regimes could greatly influence the growth rate of larvae and consequently the volume of the swimbladder.

Buoyancy of zebrafish with double-chambered swimbladders

The development of a second chamber did not result in additional buoyancy for the fish (Fig. 4). The whole-body density of larvae remained close to that for neutral buoyancy from the LSC stage through the transition into the EDC stage and even through the LDC stage into adulthood. Similar relationships between total swimbladder volume and fish growth have been reported in other teleosts.[4,37,38,47,59] Thus, by the adult stage, we found that, on average, the swimbladder contributed 4.3% of the total fish volume, a value close to that reported previously for zebrafish[32] and for other cyprinid species. Alexander[20] showed that the swimbladder occupied 5.8% (carp) and 9.9% (roach) of the total body volume in representative members of cyprinids, while subsequent work by Overfield and Kylstra[61] reported a range of 5.0%–7.1%. Alexander[2] proposed that the variation typically observed in the swimbladder volume of freshwater teleosts that maintain neutral buoyancy can largely be attributed to differences in the relative densities of different tissues, particularly bone, cartilage, and muscle.

Although the total volume of the swimbladder continued to increase linearly with zebrafish growth, even after the formation of the anterior chamber, the distribution of this volume across the two chambers changed dramatically through ontogeny in this study. In the EDC stage, the anterior chamber averaged about twice the volume of the posterior chamber. The mean ratio between the volume of these chambers then more than doubled to 4.50 at the LDC stage but upon reaching the adult form, the volume was nearly equally distributed between the two chambers (mean ratio of 1.15:1.00, anterior:posterior). Given that pressures in the two chambers were likely to have been similar,[32] such changes in the volumes of the two chambers may be a consequence of the development of components of the swimbladder walls, which might contribute to differences in the compliances of the two chambers. Indeed, by the adult stage, the swimbladder walls have developed numerous smooth muscle fibres,[31] which are not yet present at single chamber stages. The maturation of these muscles, along with addition of tissue layers containing collagen and elastin fibres,[30,33] presumably affects the distribution of gas volumes within the swimbladder system. Given these dramatic differences in the volume distribution along the horizontal body axis reported here, future studies might investigate whether they compensate for concomitant shifts in body mass, or whether they result in slight systematic changes in the pitch at which the larvae swim at different ages.

Changes in swimming depth during development

The initial inflation of the swimbladder was correlated with the subsequent ability of the larvae to swim freely near the surface of the water throughout ESC and LSC stages (Fig. 7). In the wild, this position in the water column may both incur costs and provide benefits to young larvae. For instance, the shallow position of young larvae might greatly increase their vulnerability to predators, such as dragonfly larvae, that are abundant in the natural environment of zebrafish.[46] However, larvae may also benefit from this position through their proximity to food items at the surface of the water at a time when the yolk sac is becoming depleted.[62]

During early larval development most fish are confronted with high drag and viscous forces during movement in the water column.[63] Studies of zebrafish have confirmed that larvae with lengths of 3.9–6.1 mm spend 98% of time subjected to viscous and intermediate hydrodynamic regimes compared with inertial regimes, which severely limit swimming behavior.[64] Additionally, the predominance of white muscle at this period of larval development cannot sustain continuous swimming.[64] Given these factors, it is unlikely that ESC larvae would be able to spend large periods of time swimming near the surface if they had not attained near neutral buoyancy via swimbladder inflation.

Following swimbladder inflation, zebrafish were generally neutrally buoyant across all subsequent stages of development. In contrast, mean swimming depths varied, with LDC and adult fish swimming deeper in the water column than ESC and LSC larvae. We suggest that this downward movement was due primarily to increased swimming ability of the older fish. During development, the most rapid changes in the swimming performance capability of zebrafish have been documented at body lengths between 5 and 15 mm.[64] This period of growth corresponds closely with the first occurrence of increased swimming depth noted at the EDC stage (Fig. 7), at which time larvae had a mean total length of 7.4 mm (Table 2). Further, overall cyprinid development over the first 3–4 weeks after hatching parallels a series of functional changes in swimming muscles, which gradually lead to

the establishment of the adult pattern of red, pink and white muscle fibers.[65] Power for swimming propulsion is provided by myotomal musculature which, in zebrafish, begins to develop rapidly as adult-type muscle fibers when larvae reach body lengths of 8–10 mm, and is completed by 12 mm.[64] Accordingly, at this time, zebrafish should be capable of short forays to deeper waters, with the endurance to return to shallow depths as needed.

The onset of changes in locomotory function also correlates with maturation of the swimbladder itself. Transition from the EDC to the LDC is marked by narrowing of the ductus communicans to functionally isolate the chambers, thus permitting regulation of individual chamber volumes. Robertson *et al*.[33] showed that the organization of smooth muscle bands on the posterior wall does not mature until the LDC stage, despite earlier development of vasculature and innervation. Transition from the LDC to adulthood is marked by a decrease in the relative volume of the anterior chamber, perhaps because of maturation of the structural elements of the wall leading to changes in its compliance. Thus, although older fish are capable of swimming to greater depths (at least within a limited range) and residing deeper in the water column without concomitant changes in buoyancy, they may also be capable of regulating their buoyancy via adjustment of swimbladder volume by this stage. Further, long-term regulation of buoyancy may, for example, occur with circadian rhythmicity or in response to persistent stressors causing alarm reactions. Future research must examine such possibilities.

Conclusions

This study is the first to describe the contribution of the swimbladder to buoyancy and swimming depth from first inflation to the development of the adult organ in a small teleost species, the zebrafish (*Danio rerio*). The results of our detailed examination of the behavior leading to swimbladder inflation has suggested that the extensive use of the term "swim-up" for such behavior across many teleost species may be misleading. At least in the case of zebrafish, in which larval body density is considerably greater than that of the aquatic environment, this process is characterized primarily by movement of larvae along the substrate and attachment to vertical surfaces, with only brief periods when larvae are mobile in the water column.

The morphometric approach used in this study to calculate whole-body density and swimbladder volume has provided strong evidence that, following initial inflation, the volume of gas in the zebrafish swimbladder is sufficient to compensate for the density of body tissues and allows larvae to attain near neutral buoyancy throughout all stages of development. However, our data have revealed that the range of swimming depth varies with stages of swimbladder development. Yet the mechanisms responsible for the changes in swimming depth that we observed during the transition from a single to double-chambered organ remain obscure. Additional hypotheses must therefore be formulated and tested to determine whether changes in depth result from the maturation of swimming capacity in zebrafish, the onset of functional control of the swimbladder, possible environmental factors, or a combination of these. Future field studies will be essential to compare the changes in depth during swimbladder development presented here with the vertical position of zebrafish in their native habitat and how differences in depth during ontogeny might impinge on the lifestyle of these fish in the wild.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Roger P. Croll, Ph.D.
Department of Physiology and Biophysics
Dalhousie University
5850 College St.
Halifax B3H 1X5
Canada

E-mail: roger.croll@dal.ca