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RESEARCH ARTICLE

Swim bladder morphology changes with female reproductive state in the mouth-brooding African cichlid *Astatotilapia burtoni*

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ABSTRACT

Mouth brooding is an extreme form of parental care in which the brooding parent carries the developing young in their buccal cavity for the duration of development. Brooding fish need to compensate for the brood weight on the anterior portion of their body. For fishes with a compartmentalized swim bladder, gas distribution between the chambers may aid in regulating buoyancy during brooding. To test this hypothesis, we took radiographs of *Astatotilapia burtoni* to compare the swim bladder morphology of gravid, mouth-brooding and recovering females. Following spawning, females carry developing fish in their buccal cavity for ∼2 weeks, resulting in a larger and rounder anterior swim bladder compartment. Comparatively, the swim bladder of gravid females is long and cylindrical. Using small beads to mimic brood weight and its effects on female buoyancy, swim bladder changes were induced that resembled those observed during brooding. Immediately after releasing their fry, brooding females swim at a positive angle of attack but correct their swimming posture to normal within 5 min, suggesting a rapid change in swim bladder gas distribution. These data provide new insights into how swim bladder morphology and swimming behavior change during mouth brooding, and suggest a compartmentalized swim bladder may be a morphological adaptation for mouth brooding.

KEY WORDS: Buoyancy, Cichlidae, Gas bladder, Maternal care, Teleost

INTRODUCTION

Mouth brooding has evolved independently in several animals, and is characterized as an extreme form of parental care in which a parent holds the developing young inside the buccal cavity. In fishes, this brood time can range from a few days (*Betta* spp.: Forselius, 1957; Oppenheimer, 1970) to greater than 2 months (*Bagre marinus*: Gudger, 1918; Oppenheimer, 1970). During the parental phase, the brooding parent undergoes many behavioral and physiological changes, such as forced starvation (Oppenheimer, 1970), increased energy demand (Ostlund-Nilsson and Nilsson, 2004; Reardon and Chapman, 2010), and modification of mouth activities (e.g. churning to move around brood, yawning: Oppenheimer, 1970). If the developing fish are negatively buoyant, the brooding parent must also account for the rapid weight redistribution immediately after spawning (i.e. weight of eggs in abdomen moves to mouth), which causes postural swimming changes. As the yolk-sac larvae continue to grow into fry (capable of feeding), the weight load also increases within the buccal cavity. Thus, the brooding parent needs to compensate for this changing and fluctuating weight on the anterior portion of their body.

One proposed compensatory mechanism to adjust for fluctuations in the parental fish's buoyancy during mouth brooding involves changes in respiration and gas reabsorption by the swim bladder (Oppenheimer, 1970). The swim bladder is a gas-filled structure located between the body cavity and vertebral column of the fish. While swim bladder morphology is highly variable across fishes, two major classifications exist. The physostomous swim bladder has a pneumatic duct connection between the swim bladder and gut that allows fish to 'gulp' air to inflate the swim bladder or expel air to deflate it (Hoar, 1937; Hunter, 1976). In physoclistous swim bladders, which exist in over two-thirds of all teleosts, especially more derived species (Moyle and Joseph, 2004), the pneumatic duct either never develops or atrophies after initial bladder inflation (Doroshev and Cornacchia, 1979). For example, in two species of cichlid, *Oreochromis mossambicus* and *Hemichromis bimaculatus*, no pneumatic duct is present (Doroshev and Cornacchia, 1979; McEwen, 1940). Instead, inflation is monitored by columnar epithelia in the primordial swim bladder, such that gas exchange at the thin membrane allows fish to regulate the amount of gas in the bladder (Copeland, 1969; Fänge, 1983). Most physoclistous swim bladders also contain multiple compartments separated by a diaphragm that contains a perforation in the center (Fänge, 1983; e.g. butterflyfishes: Webb and Smith, 2000; Webb et al., 2006; tilapia: Longrie et al., 2009; toadfish: Fänge and Wittenberg, 1958). Because the swim bladder is thought to be involved in regulating buoyancy during mouth brooding, having multiple compartments may allow fish better control of their swimming posture, but this remains untested.

Fish energetic studies show that fishes spend most their time swimming slowly and performing small maneuvers to correct posture and position within the water column (Nursall, 1958; Tang et al., 2000; Webb, 1994). These behaviors, rather than fast swimming, actually dominate their energy budgets. Because fish are constantly under various hydrodynamic forces, maintaining their posture and position in the water column can be costly. So, to minimize energetic costs, it is of the upmost importance to have mechanisms that correct for posture changes associated with mouth brooding (Webb, 2002).

Here, we examined how swim bladder morphology changes during mouth brooding, the most common form of parental care in cichlids (Goodwin et al., 1998), in a fish with a physoclistous swim bladder containing two compartments separated by a diaphragm. The African cichlid *Astatotilapia burtoni* is a maternal mouth-brooding fish. During spawning, females deposit their eggs on the substrate before immediately taking them into their buccal cavity. Males then present their anal fin to the female, and she nips at the egg dummies on this fin while he releases sperm to fertilize the eggs

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in her mouth. Females then spend ∼14 days incubating the developing embryos and larvae in their mouths. At the time of release, their broods can constitute up to 30% of the mother's body weight. As the developing fish are negatively buoyant, they affect the overall buoyancy and distribution of buoyant forces acting on the mouth-brooding female. Specifically, we examined how the size and shape of the anterior and posterior swim bladder compartments change across the female reproductive cycle. By attaching small weights to the underside of the buccal cavity to mimic the brood weight, we show that the weight alone is enough to induce size and shape changes in the *A. burtoni* swim bladder. We also demonstrated that fish can correct their swimming posture upon rapid weight redistribution (i.e. egg uptake or fry release) and fluctuations in buoyancy, suggesting that changes in swim bladder morphology occur within minutes. This ability to maintain position within the water column and proper swimming posture during the female reproductive cycle is critically important for both parent and offspring survival. Because mouth brooding has evolved independently several times, we hypothesize that some species, like *A. burtoni*, may have co-opted a compartmentalized swim bladder to assist in regulating buoyancy during brooding.

MATERIALS AND METHODS

Experimental animals

A laboratory-bred population of *Astatotilapia burtoni* (Günther 1894), derived from a wild-caught stock from Lake Tanganyika, Africa, was housed in aquaria under environmental conditions that mimicked their natural habitat (28°C, 12 h light:12 h dark cycle, full-spectrum illumination). Community tanks (30–100 l^{-1}) contained gravel-covered bottoms and halved terracotta pots for territorial shelters. Fish were fed cichlid flakes (AquaDine, Healdsburg, CA, USA) each morning and supplemented with brine shrimp twice weekly.

Experimental animals were collected based on reproductive state (mean±s.d. standard length: 40.707±6.499 mm; body mass: 1.661±0.730 g; N=58 fish total). Female *A. burtoni* follow a cyclic reproductive cycle with three primary phases: gravid, mouth brooding and recovering. Gravid females were selected based on the observation of an enlarged abdomen due to large, ready-to-spawn eggs and were actively being courted by males. Onset of brooding was marked daily based on the presence of eggs in the female mouth, and brooding females were collected at early (1–3 days post-fertilization, dpf), middle (6–8 dpf) and late (12–14 dpf) brooding stages. Recovering females were collected 7–21 days after the release of their brood, during which time they undergo ovarian recrudescence and vitellogenesis for their next spawning event. Following radiographs, fish were measured for standard length and body mass (M_b), and the gonads were removed and weighed (M_g) to calculate gonadosomatic index [GSI=(M_g/M_b)×100]. GSI values were used to verify reproductive state. All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2011. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA.

Radiographs and image acquisition

To visualize swim bladder morphology, we used radiographic imaging of freshly killed animals. Fish were immersed in 0.1% benzocaine prepared in fish water (reverse osmosis water supplemented with Lake Tanganyika buffer and salts; Seachem, Madison, GA, USA) and immediately placed on ice until imaging (<10 min). Lateral radiographs were taken using a Faxitron (Tucson, AZ, USA) Cabinet X-ray system at 32 kV for 1 min, developed for 2 min, rinsed in cold water for 15 s and fixed for 7 min, and all processing was performed in a darkroom. Following fixation, films were rinsed under cold water for 10 min and hung to dry overnight.

Radiographic films were backlit and photographed using an Amscope FMA050 microscope (Irvine, CA, USA) in a darkroom. All images were taken at the same magnification and exposure settings. Contrast and brightness were adjusted in Adobe Photoshop CS6 to enhance definition of the swim bladder. To isolate the swim bladder shape for morphometric analysis (see below), the swim bladder was outlined by an observer blind to the animal's reproductive state and the shape was filled. All images were aligned using the vertebral column and sagitta (largest inner ear otolith).

Mimicking brood weight

To test whether changes in swim bladder morphology were due to the weight of the developing young during mouth brooding, we mimicked brood weight using a small weight attached to the underside of the mouth of non-brooding females. We determined brood weight at various stages (days 1–3, 6–8 and 10–14; N=3–10 fish per time) by weighing the brooding female with her brood in the mouth and immediately after the brood was removed from her buccal cavity; the difference between the two was equal to the weight of the brood. Recovering females were netted from tanks, anesthetized in ice water, and a small weight (i.e. glass pony bead) approximating 10–15% of their body weight (i.e. the weight of a brood on ∼day 7 of mouth brooding; mean±s.d. 13.87±2.032% of body weight) was attached to the underside of the buccal cavity. Weights could not be placed inside the buccal cavity, as this interfered with respiration. A polyester string was sewn through both mandibular lateral line canals, the bead was centered on the underside of the fish and secured in place. Sham-handled fish underwent the same surgery, but no weight was attached to the string. Fish were placed back into their tank for 4 h prior to imaging as described above. A longer time frame was unnecessary because swim bladder gas distribution changes rapidly, and this shorter time period minimized stress to the fish.

We chose to simulate brood weight using small glass beads, which are negatively buoyant. However, we acknowledge the beads do not fully represent the buoyant forces created by broods. Both broods and beads are denser than water, thus causing the brooding females to be more negatively buoyant. The buoyant force of a liquid acting on an object is calculated by multiplying the volume of the liquid displaced by the object by gravitational acceleration and density of the liquid. Both the gravitational force and density of the water are constant, so in this case, the buoyant forces are directly proportional to the volume of the water displaced by the object. Calculating the actual buoyancy of a brooding female is problematic, as measuring the true volume of the entire brood is difficult. Measuring the volume of displaced water by a brooding female was not possible, as brooding females often release their broods during handling. As the female broods the developing fish in her buccal cavity, their volume is not additive with her own, but only increases it slightly as the buccal cavity expands throughout brood development. Instead, her weight increases as the weight of the brood increases, and this weight is easily mimicked using the above methods.

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Swimming posture measurements during swimming

To observe how changes in weight distribution (i.e. fry release) affect swimming behavior, we measured fish posture during swimming in late-stage brooding females, 12–14 days after fertilization (when eggs were taken up in the mouth) following fry release. We measured angle of attack (pitch only) because true body angle encompasses both pitch (angle on the *x*-axis) and yaw (angle along the *y*-axis). Each female was analyzed at three separate times: pre-release, immediately after fry release (within 1 min) and 5 min post-release. Fish were placed in a long, thin compartment (2×30 cm) with 5 cm of water so that they could swim only horizontally. Fish were videotaped to include at least five different horizontal swims at each time point. The body angle was measured by drawing a line from the dorsal lip to the center of the base of the caudal fin. The angle was measured between this body line and a straight reference line on the aquarium for five randomly chosen individual frames and averaged for each time point.

Morphometric analysis

Swim bladder area in lateral view was calculated in ImageJ (https://imagej.nih.gov/ij) and the shape described using the Momocs package in R (Bonhomme et al., 2014). Isolated swim bladder shapes were loaded into R, and the outline was extracted with 500 coordinates per image to create a continuous, smooth line. Procrustes superimposition was used to standardize size followed by an elliptical Fourier analysis with 18 harmonics. Finally, we used a principal component analysis (PCA) to extract the primary two components (PC1 and PC2) driving the differences in swim bladder morphology. To further analyze how swim bladder shape changes across the female reproductive cycle, we used vector deformation diagrams. The average shape of the swim bladder at each female reproductive state was determined in Momocs, and the direction and magnitude of change (i.e. vector deformation diagrams) between two sequential reproductive states (e.g. from gravid to early brooding) was calculated for ∼200 coordinates for each comparison. The number of coordinate values used for outline extractions and vector deformation diagrams was determined by the Momocs package.

To calculate swim bladder size, the anterior and posterior compartments were outlined separately, and the area tool in ImageJ was used to calculate swim bladder area of each compartment (in mm^2). A ratio of anterior to posterior compartment size was created by dividing the anterior area by the posterior area. The total swim bladder area was calculated by adding the area of the anterior and posterior compartments. We were unable to calculate swim bladder volume because of technical difficulties in obtaining dorsal and ventral radiographs. Because the swim bladder lies on an incline within the fish (rostrally the swim bladder is more dorsal than it is caudally), radiographs in dorsal and ventral view did not provide enough resolution to consistently and reliably distinguish swim bladder borders.

Statistical analysis

All statistical analyses were performed in R and SPSS. Changes in swim bladder size across the reproductive states were analyzed using an ANCOVA with standard length as a covariate. Because a Procrustes superimposition was used to standardize swim bladder images during shape analysis, body size corrections were not needed. A MANOVA on the first two principal components and ANOVA on each individual component coordinate values were used to test for differences in swim bladder shape. Student's *t*-tests were used to compare swim bladder size and shape between brood-mimicked and sham-handled fish. A repeated-measures ANOVA was used to examine changes in swimming angle due to fry release. Normality and equal variance were examined prior to testing, and transformations (e.g. log, natural log) were used when appropriate. If data did not meet normality after transformation, non-parametric tests were used and are indicated in the text. *Post hoc* comparisons were done using Tukey's test. Bonferroni or other similar corrections were not used because they increase the chance of type II errors, and their detrimental effects on statistical power can mask potential biologically relevant results, especially in small sample sizes (Nakagawa, 2004).

RESULTS

Astatotilapia burtoni has a compartmentalized (i.e. two-chambered) swim bladder similar to that previously described in the closely related Nile tilapia, *Oreochromis niloticus* (Longrie et al., 2009). The larger anterior compartment is anteriorly bilobate, with one lobe on either side of the vertebral column, and extends close to the neurocranium. In *O. niloticus*, the anterior and posterior compartments are separated by a diaphragm that is perforated by a sphincter containing both circular and radiating muscle fibers (Longrie et al., 2009). A similar perforated diaphragm separates the compartments in *A. burtoni*, presumably allowing movement of gas between the two chambers.

Radiographs were taken of six females in each reproductive and brooding state and analyzed for swim bladder shape and size (Fig. 1A). The overall size of the swim bladder (measured in lateral view) did not vary with female reproductive state (Fig. 1B; ANCOVA, $f_{4,25}$=0.898, P=0.480). In contrast, the relative size of the anterior to posterior compartments (i.e. gas distribution within the swim bladder) did change during the female reproductive cycle (Fig. 1C; ANCOVA, $f_{4,25}$=4.013, P=0.012). A larger size ratio indicates a larger anterior compartment relative to the posterior compartment. Recovering and gravid females had a low size ratio (mean±s.d.; gravid: 3.610±1.034; recovering: 5.368±1.604). However, upon egg uptake after spawning, the size ratio increased, and continued to increase during the course of brooding (early: 5.238±1.213; middle: 6.517±3.584; late: 9.486 ±2.221). Late-stage mouth-brooding females had a significantly larger anterior compartment than gravid (P<0.001), recovering (P=0.021) and early brooding (P=0.017) females. In addition, the gas distribution within the swim bladder compartments was positively correlated with day (spawning=day 0) in the female reproductive cycle (Pearson correlation; R=0.409; P=0.030).

To extract shape information, a PCA was conducted on elliptical Fourier-transformed outlines. Two primary components were extracted that explained 84.093% of the variance (Fig. 1D). PC1 was related to the angle of the swim bladder, and although there was quite a bit of variability among individuals, it was similar among all reproductive stages. PC2 was related to the shape of the swim bladder and separated females according to their reproductive stage, with a positive value being 'flat' and a negative value being 'round'. Overall swim bladder shape was different across female reproductive states (MANOVA; $f_{8,46}$=7.0902, P<0.001). Gravid females had a swim bladder shape that was significantly different from that of all three brooding groups (early: P=0.023; middle: P=0.009; late: P<0.001) but not from that of recovering females (P=0.579). Swim bladder shape in recovering females was different from that of middle (P=0.035) and late (P<0.001) brooding females, but not from that of early brooding females (P=0.069). The swim bladder shape of early brooding females was different from that of late brooding females (P=0.006) but not from that of middle

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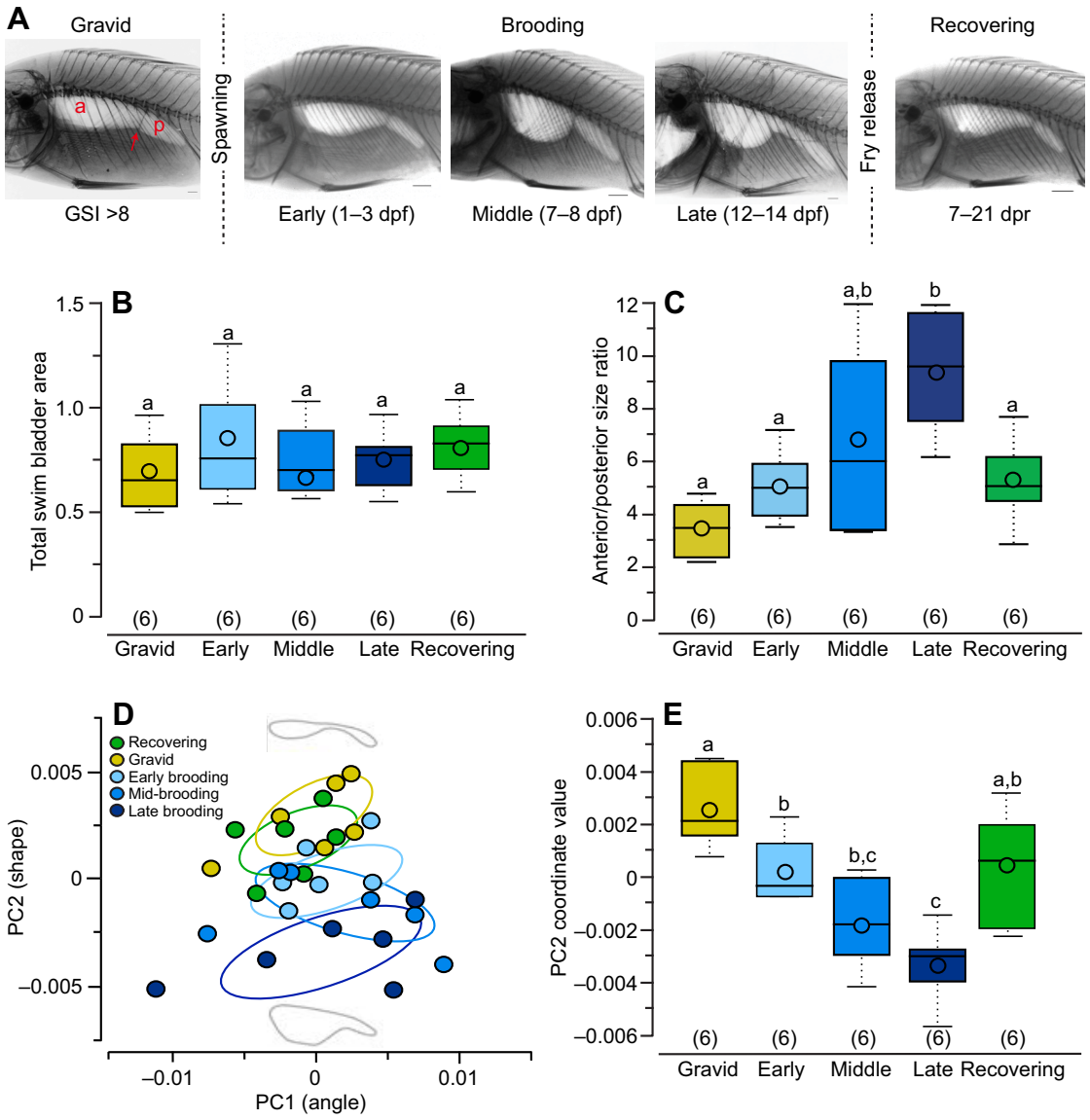

Fig. 1. Female *Astatotilapia burtoni* swim bladder morphology changes during mouth brooding. (A) Radiographs of *A. burtoni* females showing a compartmentalized swim bladder where the anterior (a) and posterior (p) compartments are separated by a perforated diaphragm (arrow). Scale bars represent 2 mm. (B,C) Overall swim bladder size does not change with reproductive state (B), but the size ratio of the anterior to posterior compartments increases during brooding (C). (D) A principal component analysis was used to examine swim bladder shape. Females in different reproductive states spread and overlap along the *x*-axis, which likely represents swim bladder 'angle', but separate along principal component (PC)2 ('roundness'). Traces in D represent the swim bladder shape of positive and negative loading values in PC2. (E) During brooding, swim bladder shape becomes more 'round', which is represented by lower coordinate values in PC2. Tukey's box plots were used to plot the data: median is represented by a line and mean by an open circle within the box; the box extends to the furthest data points within the 25th and 75th percentile and whiskers extend to the furthest data points not considered outliers. Different letters represent statistical significance at $P<0.05$. Numbers in parentheses indicate sample size. dpf, days post-fertilization; dpr, days post-release; GSI, gonadosomatic index.

brooding females ($P=0.249$), and middle and late brooding females had similar swim bladder shapes ($P=0.226$). These differences in shape were driven by PC2, which represents the 'roundness' of the anterior compartment. PC1 (swim bladder angle) coordinate values did not differ between the groups; however, PC2 coordinate values (roundness) varied with female reproductive state (Fig. 1E; Kruskal–Wallis ANOVA, d.f.=4, $h=16.861$, $P=0.002$). By the middle and late brooding stages, the swim bladder was significantly 'rounder' than that found in gravid females.

To further visualize how swim bladder shape changes across the female reproductive cycle, vector deformation diagrams were used (Fig. 2). Upon egg uptake (gravid to early brooding), the posterior compartment loses gas while the anterior compartment gains gas to increase roundness. This continued throughout brooding (early to middle brooding and middle to late brooding). Upon fry release (late brooding to recovering), gas appeared to move to the posterior compartment and the shape was more cylindrical. This continued over the course of recovery (recovering to gravid) until the female was ready to spawn again.

By mimicking brood weight with a bead approximating the weight of mid-development brood, we found that the weight alone was enough to induce changes in swim bladder shape and distribution of gas between the two compartments (Fig. 3A–C). The swim bladder compartment size ratio was larger in brood-

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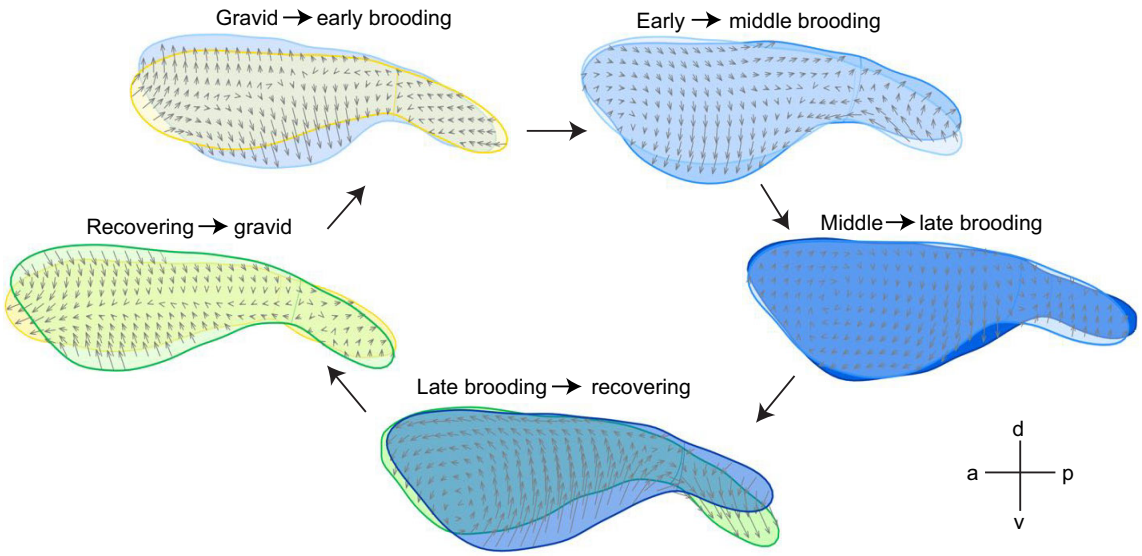

Fig. 2. Vector deformation diagrams showing how swim bladder shape changes during the female *A. burtoni* reproductive cycle. Swim bladder shapes are depicted in lateral view. Vectors represent the magnitude (length of arrow) and direction of change between swim bladder shape of each pair of sequential reproductive states for approximately 200 coordinates for each comparison. After egg uptake into the buccal cavity [gravid (yellow)→early brooding (light blue)], gas moves from the posterior compartment forward to the anterior compartment as it becomes rounder. Roundness continues to increase during early→middle (blue), and middle→late (dark blue) brooding. Upon fry release [late brooding→recovering (green)], gas returns to the posterior compartment and the shape becomes more streamlined. This continues during recovery (recovering→gravid). a, anterior; d, dorsal; p, posterior; v, ventral.

mimicked fish than in sham-handled fish (*t*-test, t_{14}=−5.415, P<0.001; Fig. 3D), indicating a larger anterior compartment. Further, this size ratio was similar to that of the middle brooding females (t_{12}=0.0531, P=0.959) that the bead was intended to mimic. The shape of the swim bladder changed to a lesser degree and was not significantly different between the sham-handled and brood-mimicked fish (P=0.234). A discriminant function analysis of swim bladder shape grouped brood-mimicked fish with brooding females, while sham-handled fish grouped with recovering females (Fig. 3E). Vector deformation diagrams indicated that the shape change between brood-mimicked and sham-handled fish was similar to the shape change from gravid to early brooding (Fig. 3F), suggesting that the swim bladder shape changes observed upon spawning and egg uptake were due to the weight of the brood.

To test how changes in weight distribution (i.e. fry release) affect swimming posture, we measured body angle during swimming after females had released their fry (Fig. 4). Late-stage brooding females swam at an angle of −1.703±1.034 deg. Immediately after releasing their fry, their swimming angle increased to 6.859±2.274 deg (mean±s.d.) but returned to −1.691±0.961 deg at 5 min post-release (repeated-measures ANOVA; $f_{6,2,12}$=21.555, P<0.001; pre-release versus immediate P<0.001; immediate versus post-release P<0.001; pre-release versus post-release P=1.00). Together with radiographs from brood-mimicked fish, these data indicate that the changes in weight distribution related to fry release and egg uptake are likely

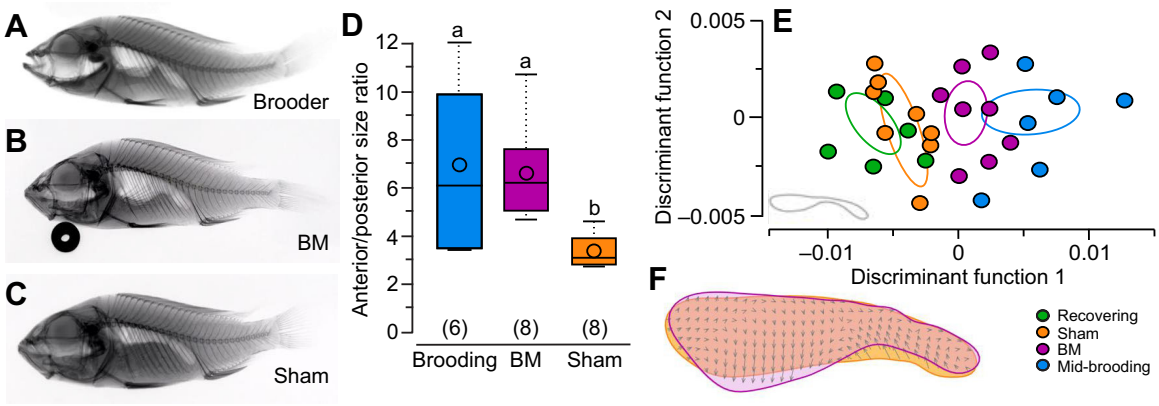

Fig. 3. Mimicking brood weight induces changes in female *A. burtoni* swim bladder size and shape. (A–D) Swim bladder size and shape were compared using radiographs of brooding (A), brood-mimicked (BM; B) and sham-handled (Sham; C) females. Brood mimicking was done by attaching a small bead underneath the mouth that approximated ∼15% of body weight, while sham-handled fish underwent the same surgery with only a string (no bead) attached. Brood-mimicked fish had a larger anterior to posterior swim bladder size ratio than sham-handled females (D). (E) Discriminant function analysis revealed that the brood-mimicked fish grouped more closely with brooding females while sham-handled fish were more similar to recovering females. (F) Vector deformation diagrams indicate that these shape changes (increase in 'roundness' of anterior compartment) between brood-mimicked (purple) and sham-handled (orange) females were due to decreased gas in the posterior compartment and an increase in roundness of the anterior compartment. See Fig. 1 legend for boxplot descriptions. Different letters represent statistical significance at P<0.05.

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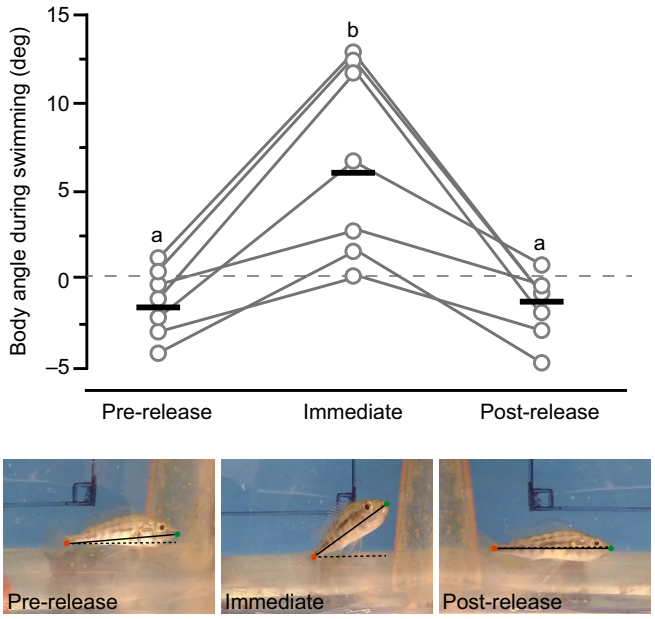

Fig. 4. Female *A. burtoni* rapidly correct their swimming posture after fry release. Following fry release, swimming posture changes such that females swim at an upward angle (positive) but return to neutral after 5 min. Each circle represents a single fish, values for individual fish are connected with lines, and thick horizontal lines represent the mean of each group. Representative photos are shown of females pre-release, immediately after release and 5 min post-release. To calculate swimming angle, a line between the base of the caudal fin (red dot) and dorsal lip (green dot) was compared with a reference line in the tank for five horizontal swims at each time point per fish. Different letters represent statistical significance at $P<0.05$.

compensated for by changes in swim bladder morphology to regulate buoyancy and control posture for swimming.

DISCUSSION

We have shown for the first time that swim bladder morphology changes with female reproductive state in a mouth-brooding fish species. The change in morphology allows females to maintain the proper buoyancy and swimming posture necessary for survival. In *A. burtoni*, females carry their broods for 12–14 days. Their broods can weigh up to 30% of the total body weight of the parent, which negatively impacts parental buoyancy. Using radiograph imaging, we showed that total swim bladder area does not change during brooding, as previously suggested (Oppenheimer, 1970). Rather, we demonstrated that *A. burtoni* regulates gas distribution between the anterior and posterior swim bladder compartments through a perforated diaphragm. In females, the anterior chamber comprises 50–90% of the total swim bladder size. We analyzed gas distribution inside the swim bladder by creating a ratio of the size of the anterior to the posterior compartments. As the brood develops and increases in weight, the brooding female's swim bladder gas distribution changes in a similar manner (Fig. 5). Brood weight increases most dramatically during middle brooding days (6–8 dpf). This corresponds to the time period when high variation in female swim bladder morphology also occurs, suggesting that this bladder variation may be due to variability in weight, and its impact on the female's buoyancy, of the growing brood. The shift in gas distribution observed in middle and late brooding females indicates that brooding fish have more gas in the anterior compartment of their swim bladder, likely to act as a counterbalance to the weight of the developing young inside the buccal cavity.

Based on our results, we propose the following mechanism for how these swim bladder changes occur. In many physoclistous species with a compartmentalized swim bladder, the anterior compartment has secretory tissue in its lining, while the posterior compartment contains absorbent tissue (Fänge, 1983). Although not examined in *A. burtoni*, the swim bladder diaphragm in the closely related tilapia *O. niloticus* contains circular and radiating smooth muscle fibers to allow for constriction and dilation of a sphincter to regulate gas flow between the two chambers (Longrie et al., 2009). *Astatotilapia burtoni* has a similar diaphragm, and we propose that this sphincter is constricted in brooding females to keep more gas in the anterior compartment. Upon release of fry, the sphincter is relaxed and allows gas to rapidly redistribute between the two chambers. Because gas is secreted into the anterior compartment (Fänge, 1983; Wittenberg et al., 1964), and by reducing gas flow into the posterior compartment, the anterior compartment increases in size while the posterior compartment decreases in relative size. This mechanism mediates gas distribution within the swim bladder during the female reproductive cycle, thereby compensating for brood-induced changes in weight distribution in the mouth. Neither brooding nor mimicking brood weight resulted in any changes to the total swim bladder area. This is possibly an artifact from two-dimensional measurements of the lateral swim bladder area rather than volume in all three dimensions. Alternatively, as fish were kept in shallow-water tanks (maximum depth ~30 cm) and mouth-brooding females primarily stay at or near the bottom of the water column, they may not have needed to adjust their total swim bladder volume and overall buoyancy in our experiments.

Oppenheimer (1970) notes in the blackchin tilapia, *Sarotherodon melanotheron*, that the brooding parent increases mouth activity (e.g. churning), which potentially increases water flow over the gills to improve respiration and gas absorption by the swim bladder once

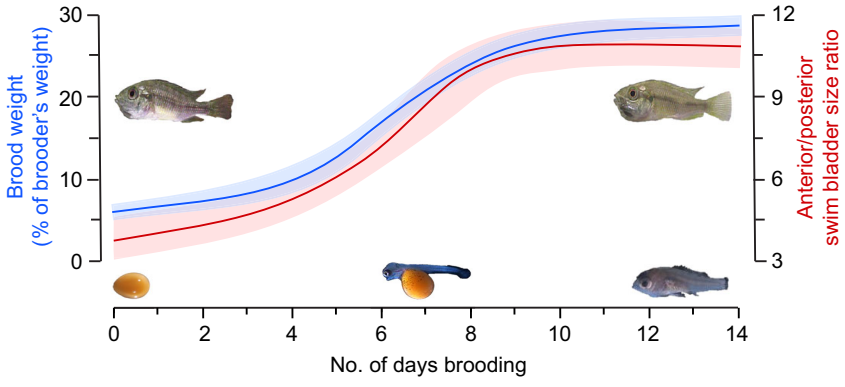

Fig. 5. Brood weight and brooding female swim bladder morphology change in similar patterns in *A. burtoni*. As brood weight (blue) increases during development, gas distribution within the swim bladder (ratio of anterior to posterior compartment size, red) also increases. The most rapid change for both brood weight and female swim bladder size ratio occurs during the middle brooding days (6–8 days post-fertilization). Means±s.d. are indicated for each line based on values collected on days 0–3, 6–8 and 10–14 for brood weight, and days 1–3, 6–8 and 12–14 of brooding for female swim bladder size ratio. Representative photos are shown of early brood (upper left) and late brood (upper right) females and embryo/eggs (bottom left), yolk-sac larva (bottom middle) and transformed juvenile (bottom right) young across the brooding cycle.

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the fertilized eggs are in the buccal cavity. This further supports the idea that gas is preferentially added to the anterior chamber during brooding. Alternatively, changes in swim bladder shape could be a result of available space within the abdominal cavity. Gravid females have large ready-to-spawn eggs in their ovaries and are consuming food, leading to full stomachs and intestines. With these organs taking up more space, the swim bladder may be forced into a more streamlined shape. However, this scenario is unlikely in *A. burtoni* because recovering females, which have small ovaries, also have a streamlined swim bladder shape which is similar to that of gravid individuals. In addition, the ovaries are largest in the posterior portion of the abdominal cavity, suggesting that if their size impacted swim bladder shape, it would force more gas to the anterior compartment and not the posterior compartment as observed here. Although several potential mechanisms exist for how and why swim bladder morphology changes, our results are consistent with the hypothesis that these changes are directly related to the weight of the developing brood in the buccal cavity.

Developing fish are thought to be neutrally buoyant after hatching (Oppenheimer, 1970), but the swim bladder does not inflate for several days after hatching (Doroshev and Cornacchia, 1979). In our population of *A. burtoni*, hatching occurs at 3–4 dpf, at which time the fry are not neutrally buoyant. Free-swimming *A. burtoni* fry do not appear to be buoyant until at least 12 dpf, and radiographs of developing and newly released transformed juveniles show that they do not yet have an inflated swim bladder (personal observations, J.M.B.). As such, the developing brood acts as a weight in the brooding female's buccal cavity for the duration of development. The negative buoyancy of the brood affects not only the overall buoyancy of the brooding female but also the distribution of buoyant forces acting on the female. Recovering females with an attached bead approximating a mid-development brood had a similar gas distribution pattern to middle-stage brooding females. However, females that underwent the sham surgery retained a recovering female-like swim bladder. In a discriminant function analysis of swim bladder shape, brood-mimicked females grouped together with brooding females while sham-handled females grouped together with recovering and gravid females, indicating that although not significantly different, brood weight is enough to induce swim bladder shape changes. Although we mimicked brood weight instead of brood buoyancy, the bead and brood had similar effects on the female. Future work is needed to fully understand how mouth brooding affects the buoyancy of brooding fish.

Changes to the swim bladder in female *A. burtoni* also occurred relatively rapidly. During brood mimicking, 4 h with weight approximating a mid-development brood was enough to induce changes in the gas distribution and shape of the swim bladder. Upon fry maturation (~14 dpf; yolk-sac fully absorbed), the brooding parent will open her mouth and release her brood, causing a rapid decrease in her weight and increase in buoyancy, particularly in the buccal region. Many brooding cichlids also offer some form of protective parental care after the fry have been initially released, by collecting the fry back into their buccal cavity when threatened (Barlow, 2002). Other fishes will collect their fry that wander away from the nest into their mouths for transport back to safety (Baerends and Baerends-Van Roon, 1950; Fryer and Iles, 1972). In these examples, if the fry are not yet neutrally buoyant, they again contribute to increased weight in the brooding parent's mouth and negatively impact their buoyancy. We measured swimming angle in late-stage brooding females and found that they swam at a relatively low angle (i.e. parallel to the tank floor; −1 to +1 deg). This position allows for optimal function of sensory systems and minimizes the drag acting on the fish (Weihs, 1993). Immediately after releasing their fry, however, females swam more erratically, often with their head pointed upwards, suggesting that the anterior portion of the swim bladder was still large and more buoyant, causing them to swim at a positive upward angle (~7 deg). This increase in the angle of attack exposes the females to more hydrodynamic drag and causes them to perform more correcting maneuvers, thus expending more energy. Females corrected their swimming posture within 5 min of fry release, indicating that adjustments in gas redistribution can occur rapidly. This rapid correction would be advantageous for parental care behaviors and likely improves the survivability of both the brooding parent and their fry.

The center of mass and buoyancy occur at different locations in a fish, causing many fishes to have a tendency to roll. The metacentric height, or vertical distance between the centers of mass and buoyancy, determines the magnitude of rolling, and thus how much energy is needed to counteract this rolling force (Marchaj, 1991; Webb, 2002). As the brood develops, the center of mass of the brooding female changes. The observed changes in size and shape of the *A. burtoni* anterior compartment may change the center of buoyancy, thus affecting the metacentric height and the amount of energy to maintain posture and stability in the water column.

Computed tomography imaging was previously used to examine swim bladder morphology in a variety of fishes (Mohr et al., 2017; Schulz-Mirbach et al., 2012; Webb et al., 2006). This imaging technique can provide more detailed information on the swim bladder volume and relationship with surrounding structures (e.g. inner ear, lateral line). Unfortunately, micro-CT imaging could not be used in the current study because of post-mortem gas equilibration between the two chambers, making any relative changes between anterior and posterior compartments undetectable. Using the methodology reported here, we are confident that gas had not redistributed in the time frame used for analysis. All animals were imaged within ~10 min of collection and imaging took 1 min. Repeated imaging of the same fish 1 h after euthanasia showed that gas passively diffused between the two chambers, indicating that micro-CT imaging would not have been useful in the current study.

We used vector deformation diagrams to examine how the average swim bladder shape changed between each female reproductive and brooding state. While supporting the data on gas redistribution within the swim bladder during brooding, this analysis also revealed that the anterior lobes of the swim bladder appear to have slightly different projection angles towards the brain case and inner ear depending on reproductive state. Morphological connections between the swim bladder and inner ear enhance hearing capabilities in fishes by transducing sound pressure detected via the swim bladder into particle motions detected by the otolithic endorgans (Blaxter, 1981; Blaxter et al., 1981; Ladich, 2016; Sand and Enger, 1973). Recent research indicates that even in the absence of such morphological specializations (e.g. otophysic connection), the proximity of the swim bladder to the inner ears may be enough to enhance hearing capabilities (Schulz-Mirbach et al., 2012). As gravid and brooding *A. burtoni* females have different hearing capabilities (e.g. gravid females have higher sensitivity than brooding females) (Maruska et al., 2012), the difference in proximity of the anterior swim bladder lobes to the inner ear cavities among the reproductive states deserves further study.

Cichlids are well known for their adaptive radiation (McMahan et al., 2013; Seehausen, 2006) and diverse reproductive strategies (Barlow, 2002; Kuwamura, 1986). Further research should examine the possible evolutionary relationship between mouth brooding and swim bladder

morphology. Some brooding fishes, for example, only pick up their young after hatching (presumably when they are neutrally buoyant), while others carry the developing young for only a few days instead of weeks (Oppenheimer, 1970). These differences may place different physiological, morphological and behavioral constraints on the brooding female's need to compensate for brood weight. Future studies should investigate the selective pressures that may have shaped the relationship between mouth brooding and swim bladder morphology, as well as examine how fish lacking a compartmentalized bladder compensate for brood weight.

In summary, we have shown for the first time that swim bladder morphology changes during mouth brooding. Sexual dimorphism in swim bladder morphology was previously described (Mohr et al., 2017; Rose, 1961), but this is one of the first examples of plasticity in swim bladder morphology within a sex across the reproductive cycle. Instead of adding gas to the bladder to increase buoyancy, gas is redistributed to increase the size of the anterior compartment while decreasing the size of the posterior compartment. These changes occur rapidly, such that females can correct their swimming posture within minutes of egg uptake into the mouth during spawning or after fry release. Because *A. burtoni*, and many other mouth-brooding fishes, provide some form of parental care after fry maturity (e.g. take them back into the buccal cavity in the presence of a threat), it is especially important for their own and their offspring's survival to rapidly adjust to this fluctuating weight.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.M.B., P.C., K.P.M.; Methodology: J.M.B., S.M.W., A.P.G., P.C., K.P.M.; Software: J.M.B., P.C.; Formal analysis: J.M.B.; Investigation: J.M.B., S.M.W., A.P.G.; Data curation: J.M.B.; Writing - original draft: J.M.B.; Writing - review & editing: J.M.B., S.M.W., A.P.G., P.C., K.P.M.; Visualization: J.M.B.; Supervision: K.P.M.; Funding acquisition: P.C., K.P.M.

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