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## RESEARCH ARTICLE

Kinematics of mouthbrooding in *Oreochromis niloticus* (Cichlidae)Sam Van Wassenbergh<sup>1,2,\*</sup>, Iris Joris<sup>1</sup>, Mathieu Desclée<sup>1</sup>, Hon Jung Liew<sup>1,3</sup>, Gudrun De Boeck<sup>1</sup>, Dominique Adriaens<sup>2</sup> and Peter Aerts<sup>1,4</sup>

## ABSTRACT

Many species from several different families of fishes perform mouthbrooding, where one of the sexes protects and ventilates the eggs inside the mouth cavity. This ventilation behaviour differs from gill ventilation outside the brooding period, as the normal, small-amplitude suction-pump respiration cycles are alternated with actions including near-simultaneous closed-mouth protrusions and high-amplitude depressions of the hyoid. The latter is called churning, referring to its hypothetical function in moving around and repositioning the eggs by a presumed hydrodynamic effect of the marked shifts in volume along the mouth cavity. We tested the hypothesis that churning causes the eggs located posteriorly in the mouth cavity to move anteriorly away from the gill entrance. This would prevent or clear accumulations of brood at the branchial basket, which would otherwise hinder breathing by the parent. Dual-view videos of female Nile tilapias (*Oreochromis niloticus*) during mouthbrooding showed that churning involves a posterior-to-anterior wave of expansion and compression of the head volume. Flow visualisation with polyethylene microspheres revealed a significant inflow of water entering the gill slits at the zone above the pectoral fin base, followed by a predominantly ventral outflow passing the ventrolaterally flapping branchiostegal membranes. X-ray videos indicated that particularly the brood located close to the gills is moved anteriorly during churning. These data suggest that, in addition to mixing of the brood to aid its oxygenation, an important function of the anterior flow through the gills and buccal cavity during churning is to prevent clogging of the eggs near the gills.

**KEY WORDS:** Cichlids, Tilapia, Ventilation, Opercula, Jaw protrusion, Churning, Hydrodynamics, Biomechanics

## INTRODUCTION

Mouthbrooding is the behaviour of fishes to protect and ventilate the eggs in the buccal cavity until they have developed into free-swimming fry (Keenleyside, 1991). This form of parental care is found in at least nine families of Teleostei, including numerous species from the family of cichlids (Cichlidae) (Oppenheimer, 1970). Evolutionary transitions from close guarding of the eggs in nests or crevices to mouthbrooding have occurred at least 10 times in the history of cichlids (Goodwin et al., 1998). During mouthbrooding, the buccal cavity of the parent is brought into a typical, enlarged posture to accommodate more eggs (Oppenheimer and Barlow, 1968; Goedel, 1974). In the Nile tilapia (*Oreochromis*

*niloticus*), the model species of the current study, this posture includes a slightly protruded premaxilla, a depressed hyoid (Fig. 1A) and abducted suspensoria (Fig. 1B). X-ray pictures with a radio-opaque fluid filling the buccal cavity show how drastically the buccal volume increases by this postural change (Fig. 1C), allowing large females of this species to brood more than 1500 eggs (Valentin et al., 2015).

Ventilation behaviour of the mouthbrooding parent is crucial for the survival of the parent fish and the young (Oppenheimer and Barlow, 1968). Oxygen for the eggs and the newly hatched young needs to be supplied by the flow of fresh water generated by the cranial movements of the mouthbrooder. Not surprisingly, ventilation behaviour during mouthbrooding differs from the repertoire of cranial motions observed outside the mouthbrooding period. In cichlids, two main behaviours are displayed in bouts of varying length (see Movie 1), the percentage occurrence of which varies throughout the course of the brooding period (Oppenheimer and Barlow, 1968): (1) respiration and (2) churning.

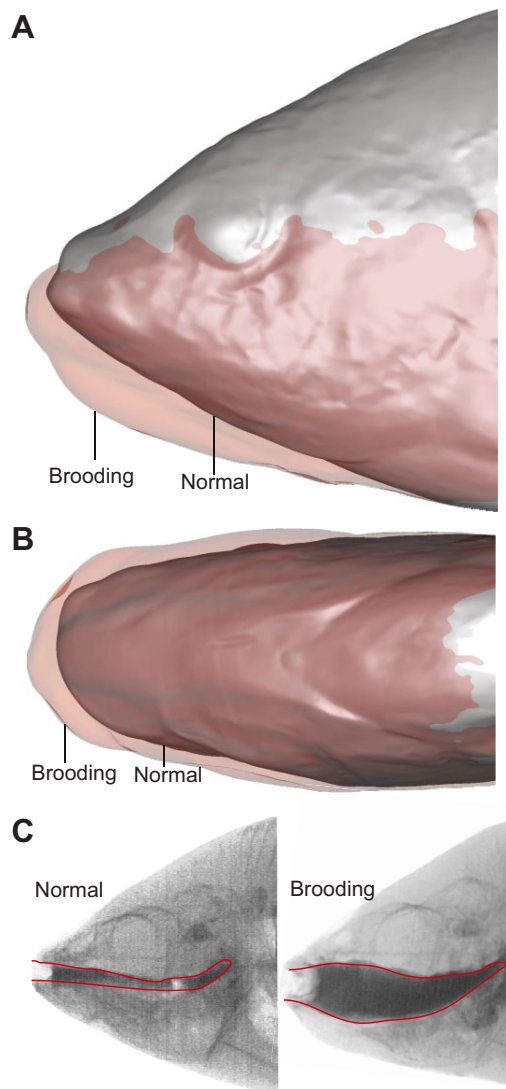
Respiration corresponds to the gill-ventilating, suction-pump movements also performed when the fish are not mouthbrooding. As a large variation exists in the amplitude of the cranial movements during respiration, a distinction is sometimes made between ‘active’ respiration (including large-amplitude motions of the jaws and opercula) and ‘passive’ respiration (involving small-amplitude jaw motions and small opening of the gill slits almost exclusively by movement of the branchiostegal membranes) (Oppenheimer and Barlow, 1968). During active respiration, Oppenheimer and Barlow (1968) described that, when the mouth is opened, the incoming water causes the uppermost eggs to move backward to the rear of the buccal cavity, while the eggs on the left side move in a clockwise manner (anti-clockwise for the eggs on the right side) and return forward along the side of the mouth (Oppenheimer and Barlow, 1968).

Churning involves a closed-mouth protrusion of the premaxilla, depression of the hyoid and abduction of the opercula (Oppenheimer and Barlow, 1968). The name of this behaviour refers to its hypothetical function in moving the brood around in the buccal cavity. At the instant during churning when the mouth is open, the eggs have been observed to roll about in the mouth (Baerends and Baerends-van Roon, 1950). Abraham (1901) peered through the semi-transparent, extended skin beneath the lower jaw of *Pseudocrenilabrus philander* and saw the hatched ‘wrigglers’ being rushed to the front of the buccal cavity, after which they retreated out of sight to the back (Abraham, 1901).

The above observations suggest that the main difference in terms of brood movement between active respiration and churning is an abrupt forward impulse that is given to the intra-oral water during churning. This forward displacement of the brood may play a role in preventing the eggs from clogging near the gills and thereby causing respiratory obstruction (Abraham, 1901). If so, churning has a role comparable to a ‘forward cough’ (*sensu* Kuiper, 1907). Coughing is regarded as a normal part of the respiratory activity of most fish (Hughes and Adeny, 1977). Alternatively, if the mouthbrooder’s

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**Fig. 1. Mouthbrooding posture of the head in the Nile tilapia.** Mimicking our video images of this species during mouthbrooding, the head of a dead specimen was forced into the mouthbrooding posture and subjected to laser scanning (A, lateral view; B, dorsal view) and X-ray imaging using a dense barium sulphate solution filling the buccal cavity (C). Red lines show the outlines of the buccal cavity.

respiration flows would not be critically hindered by the eggs, the exclusive function of churning could be to mix the eggs to reposition those deprived of sufficiently oxygenating flows. It is also possible that churning has a dual function of both mixing the eggs and preventing them from clogging near the gills.

However, the kinematics of the head parts, water and brood has thus far not been described quantitatively. This limits our understanding of the function of the alternation of respiration and churning during brooding, and how this is realised by the cranial musculoskeletal system. As the flow of water inside the mouth cavity is determined by the kinematics of the elements that influence the shape and size of the mouth cavity, as well as by the opening and closing of the potential inlets and outlets (i.e. mouth aperture and gill slits), in this study we took the first step towards a better understanding of these functional differences by comparing the cranial kinematics of respiration and churning. We then more directly evaluated aspects of the resulting flows of water during churning by flow visualisation experiments.

We aimed to answer the following three questions. (1) given the hypothesised forward intra-oral flow, is a posterior-to-anterior wave of buccopharyngeal expansion present during churning, as opposed to the normal anterior-to-posterior wave during respiration? (2) Does churning move the eggs to the front of the mouth, and is this a movement that allows a homogeneous scrambling of the eggs versus a more localised movement, focusing on the displacement of the eggs that are positioned near the branchial sieve? (3) As opening of the gill slits by opercular abduction is clearly present during churning (Oppenheimer and Barlow, 1968), does this imply an inflow of water through the gills slits? Such a flow could be useful to clear or prevent obstructions in the posterior buccal cavity resulting from egg accumulations.

## MATERIALS AND METHODS

### Animals and experimental conditions

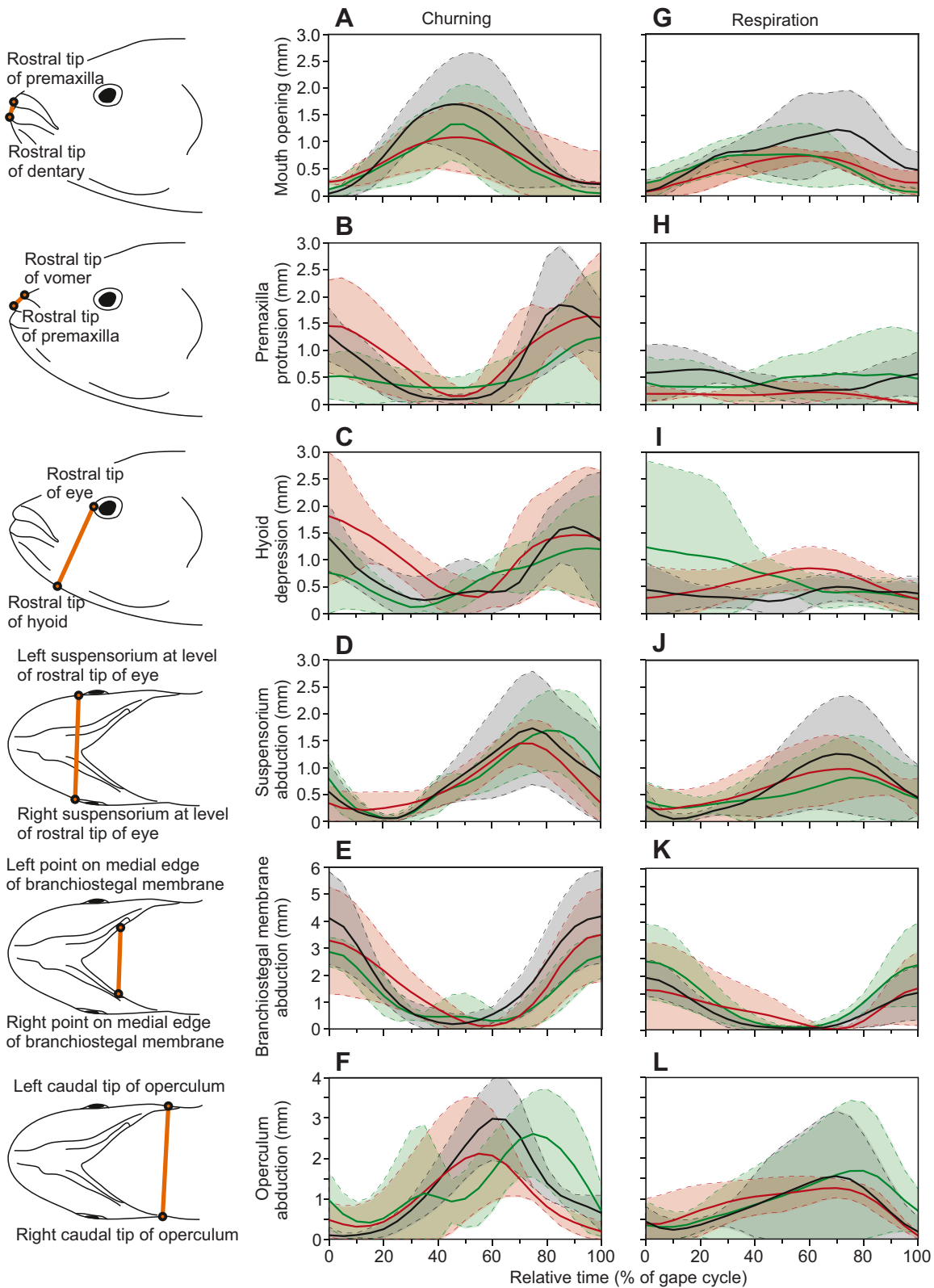
Three adult females ( $149 \pm 33$  g, mean  $\pm$  s.d.) and three adult males ( $190 \pm 29$  g) of *Oreochromis niloticus* (Linnaeus 1758) were kept as pairs in separate 120 l aquaria at  $27^\circ\text{C}$ , under a 12 h:12 h day:night cycle, and fed *ad libitum* with cichlid pellets. Males and females were separated by a grid to avoid bite wounds. When the female appeared ready to mate, the grid was removed and the male and female were united until spawning was completed. The mouthbrooding female was then transferred to a 35 l aquarium for the video-recording sessions to quantify mouthbrooding kinematics. During these sessions, the animals were gently constrained into a corner of the aquarium using grids so that the head was in the field of view of the cameras. One female of 136 g was used in the additional fluid visualisations and X-ray videos, for which it was transferred to a small aquarium with thin, radio-transparent, Plexiglas walls for the duration of the recording.

### Analysis of cranial kinematics

Mouthbrooding was filmed from a lateral and ventral view with two JVC Everio GZ-GX1 cameras (JVCKENWOOD Corporation, Kanagawa, Japan) at  $50 \text{ frames s}^{-1}$  ( $1920 \times 1080$  pixels, shutter time 0.01 s) for 20 min on day 1, 3, 5, 7 and 9 of the brooding period. Synchronisation ( $\pm 0.02$  s) between the frames from the two cameras was inferred from a LED-flash at the start of each recording. The grid used for position-constraining that was just behind the fish on the lateral-view images was used for scaling. From the 20 min of video from each of these 5 days, a single cycle of churning and ventilation was selected in which (1) the head was positioned centrally in the view of both cameras and (2) the head showed negligible roll, yaw or pitch.

The two-dimensional coordinates of 11 anatomical landmarks were determined in each video frame using Didge 2.3 (Alistair Cullum, Creighton University, NE, USA). From these coordinates, six kinematical variables were determined as distances between two of these landmarks minus the minimum distance of the entire cycle: (1) mouth opening, (2) premaxilla protrusion, (3) hyoid depression, (4) suspensorium abduction, (5) branchiostegal membrane abduction and (6) operculum abduction. The precise meaning of these variables is illustrated graphically in Fig. 2 (left). Digitisation noise was filtered on the distance versus time profiles using a zero-phase shift, fourth-order low-pass Butterworth filter with a cut-off frequency of 3 Hz.

In order to compare the kinematics of respiration with that of churning in a standardised way, each video frame was assigned a 'relative time'. The relative duration of the full motion cycle is 100%, and the start of mouth opening is set as relative time=0%. This allows calculation of the average kinematic profiles for each



**Fig. 2. Cranial kinematics of churning and respiration in three individual Nile tilapias.** (A–F) Churning; (G–L) respiration. Mean kinematic profiles ( $N=4$  or  $5$ ) per individual (colour coded) with standard deviation ranges (shaded area bordered by dashed lines) are shown as a function of relative time (% of cycle duration) for the six variables illustrated on the left.

individual ( $N=5$ , 4 and 4 for each behaviour; no data for day 5 in two of the three individuals) without generating potentially confusing time-averaged profiles with multiple peaks due to differences in

duration of the cycles in absolute time. To test whether cycle duration and the amplitude of the quantified motions differed between the two behaviours (respiration and churning), a two-way



ANOVA was used with individual as the second factor. Two variables, maximum opercular abduction and maximum protrusion, were log-transformed to pass the normality criterion (Shapiro–Wilk  $P > 0.05$ ). Equality of the variances ( $P > 0.05$ ) was always met. The interaction effect of the factors individual and behaviour was included in the model but was never significant. Statistics were performed using SigmaPlot 11.0 (Systat Software Inc., Germany).

### External flow visualisation

To visualise the inflow and outflow through the opercular slits, bright yellow polyethylene microspheres with a density of  $1.00 \text{ g cm}^{-3}$  and a diameter of 425–500  $\mu\text{m}$  (Cospheric LLC, Santa Barbara, CA, USA) were added to the water of the small filming aquarium. Close-ups of the opercular region were filmed at 250 frames  $\text{s}^{-1}$  using a Redlake M3 camera (Redlake, San Diego, CA, USA). Two LED panels (Falcon Eyes, Hong Kong) provided the necessary illumination. To describe the general pattern of flow outside the opercular slits during churning, the best video (i.e. with good image sharpness and a high number of particles in the region of interest) was selected on which 49 individual particles were tracked.

### X-ray video analysis

We managed to place a small, wooden sphere with a fragment of steel in the centre into the buccal cavity of a mouthbrooding female and record its motion during nine sequences of churning using high-speed X-ray video. This experiment was the only successful one of five attempts. In the unsuccessful experiments, the brooding females responded to either sedation (using MS222) or manipulation (forcing the dummy egg held by forceps into the mouth) by expelling all the eggs. The sphere is larger than the eggs (diameter of 4.5 mm versus approximately 2.6 mm for an egg), and has a higher density despite the positively buoyant wooden surface. However, inducing accelerated water movement in a cup containing the sphere together with a large number of eggs after the experiment showed that the sphere only minimally lagged behind the motion of the eggs, and kept its original position in the centre of the pack of eggs even after several trials. Consequently, the path of the sphere should be a good approximation of the path of the eggs in its vicinity.

Lateral-view X-ray videos were filmed at 500 frames  $\text{s}^{-1}$  with a Redlake MotionPro camera (1280×1024 pixels) attached to the image intensifier of a Philips Optimus M200 X-ray system (Royal Philips Electronics, Eindhoven, The Netherlands). The path of the sphere was determined by frame-by-frame, manual digitisation of the position of the sphere using Didge 2.3. The coordinates of two additional landmarks were determined in each video frame to recalculate the path of the sphere in a head-bound frame of reference: a landmark in the centre of the otolith (reference frame origin) and one at the anterior tip of the vomer (defining the  $x$ -axis). The  $y$ -axis was perpendicular to the  $x$ -axis and pointed dorsally.

## RESULTS

### Cranial kinematics

The average kinematic profiles of the three individuals showed a consistent pattern within each behaviour (i.e. churning and respiration) when time was scaled to cycle duration and a relative time ( $t_{\text{rel}}$ ) of 0% was set at the start of mouth opening (Fig. 2). A churning cycle lasted on average  $1.0 \pm 0.3 \text{ s}$  (mean  $\pm$  s.d.), a respiration cycle  $0.9 \pm 0.4 \text{ s}$ . Although cycle duration significantly differed between individuals ( $F_{2,1} = 4.36$ ,  $P = 0.0027$ ), it did not differ significantly between churning and respiration ( $F_{1,2} = 0.99$ ,  $P = 0.33$ ).

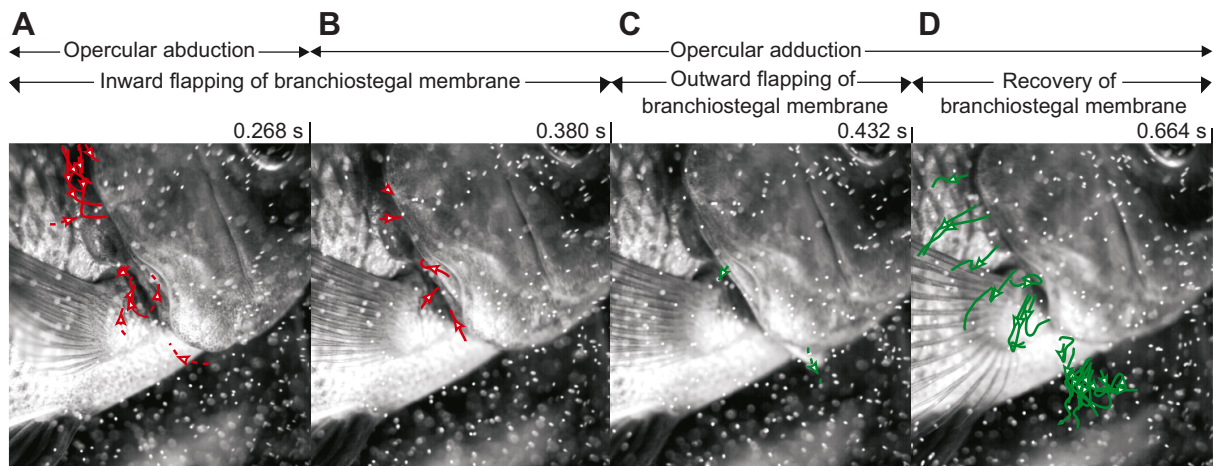
A narrow opening of the mouth was observed during churning ( $1.4 \pm 0.3 \text{ mm}$ ) as well as during respiration ( $1.0 \pm 0.2 \text{ mm}$ ), which did not differ significantly between churning and respiration ( $F_{1,2} = 1.58$ ,  $P = 0.23$ ). However, peak mouth opening was reached about halfway through the cycle duration during churning ( $t_{\text{rel}} = 48 \pm 3\%$ ), but later during respiration ( $t_{\text{rel}} = 62 \pm 8\%$ ) (Fig. 2A,G). Upper jaw protrusion was virtually absent during respiration ( $0.8 \pm 0.5 \text{ mm}$ ; Fig. 2H), but as churning was *a priori* identified when considerable protrusion occurred ( $2.0 \pm 0.9 \text{ mm}$ ), the latter was obviously significantly higher ( $F_{1,2} = 32.0$ ,  $P < 0.001$ ; Fig. 2B).

Churning involved larger expansions of the buccal and opercular cavities compared with respiration. A consistent pattern of depression of the floor of the buccal cavity at the level of the hyoid tip was only observed during churning ( $2.0 \pm 0.7 \text{ mm}$ ; Fig. 2C), not during respiration (Fig. 2I). Peak values of hyoid depression were thus significantly higher during churning ( $F_{1,2} = 8.95$ ,  $P = 0.007$ ). Also, abduction of the opercula ( $F_{1,2} = 6.78$ ,  $P = 0.017$ ; Fig. 2F,L) was significantly greater during churning ( $2.8 \pm 1.2 \text{ mm}$ ) compared with respiration ( $1.7 \pm 1.1 \text{ mm}$ ). Abduction of the suspensoria followed the same trend (churning:  $1.7 \pm 0.7 \text{ mm}$ , respiration:  $1.1 \pm 0.7 \text{ mm}$ ; Fig. 2D,J), but this difference was not great enough to exclude the possibility that it was just due to random sampling variability after allowing for the effects of differences in individuals ( $F_{1,2} = 3.98$ ,  $P = 0.060$ ).

As hypothesised, a posterior-to-anterior wave of expansion was indeed present during churning, but not during respiration. During churning, first the opercula reach their peak abduction ( $t_{\text{rel}} = 63 \pm 10\%$ ; Fig. 2F), followed by the peak abduction of the suspensoria ( $t_{\text{rel}} = 77 \pm 6\%$ ; Fig. 2D), then peak hyoid depression ( $t_{\text{rel}} = 90 \pm 5\%$ ; Fig. 2C) and finally peak upper jaw protrusion ( $t_{\text{rel}} = 93 \pm 8\%$ ; Fig. 2B). The mouth starts to close ( $t_{\text{rel}} = 48 \pm 3\%$ ) before the expansion wave starts at the posterior side of the head, and can safely be considered fully closed from a  $t_{\text{rel}}$  of about 80% onwards (Fig. 2A) when the expansion wave reaches the anterior side of the head. Lateral flapping of the ventral portion of the branchiostegal membrane was not part of this expansion wave, as it occurred later ( $t_{\text{rel}} = 98 \pm 3\%$ ) while the other elements were compressing the buccal and opercular cavity. During respiration, the instant of mouth opening ( $t_{\text{rel}} = 62 \pm 8\%$ ; Fig. 2G) on average slightly preceded the abduction of the suspensoria (Fig. 2J) and opercula (Fig. 2L; both at  $t_{\text{rel}} = 72 \pm 3\%$ ), after which abduction of the ventral part of the branchiostegal membrane occurred (peak at  $t_{\text{rel}} = 98 \pm 3\%$ ; Fig. 2K).

### Water flow at the gill slits

Position tracking of polyethylene microspheres using high-speed video showed the pattern of inflow and outflow at the opercular and branchiostegal slits illustrated in Fig. 3 and Movie 2. When the operculum is abducted, the branchiostegal membrane connected to the vertical, posterior part of the operculum edge flaps inwards towards the gills. During this phase, water is sucked towards and into the opercular cavity through the gill slit (Fig. 3A). This inward flow continued during approximately the first 0.1 s of the opercular adduction (Fig. 3B). No spheres were observed to enter at the ventral side of the gill slit below the base of the pectoral fin. Next, the branchiostegal membrane at the opercular edge flaps outwards (i.e. posteriorly away from the gills) and the first microspheres are observed to exit the gill slit (Fig. 3C). Many more spheres exited later when the dorsoposterior part of the branchiostegal membrane slowly moved inwards again. Relatively few spheres were observed exiting above the base of the pectoral fin (4 out of 18): most of these were released in a jet of rotating flow ventral to the head (Fig. 3D).



**Fig. 3.** Paths of the tracked microspheres that flow in and out of the gill slits during a representative churning sequence. Inflowing paths are shown in red, outflowing paths in green. Paths with dashed lines are continued in the next image. The sequence is subdivided into four phases (A–D) depending on the kinematics of the operculum and the dorsoposterior part of the branchiostegal membrane, as shown by the ranges indicated by the horizontal arrows at the top. The end time of each interval is shown on the upper right of the images (start of the first interval is at 0 s).

The above pattern was confirmed in many other videos that were recorded.

### Egg movement during churning

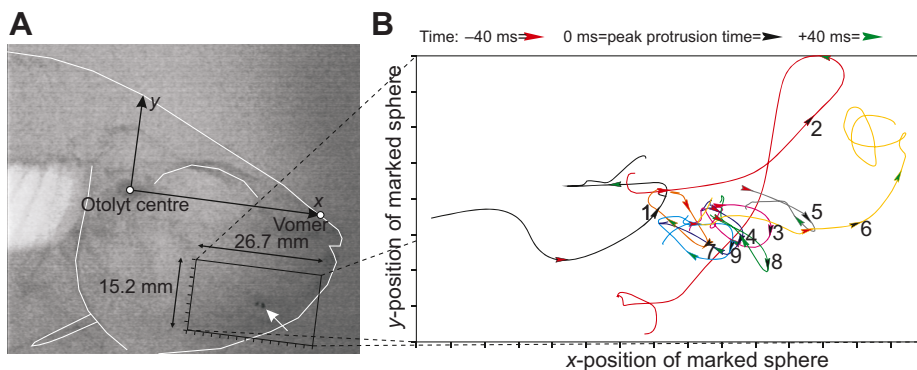
During nine churning acts of one individual, the paths travelled by a sphere, with a radio-opaque centre, inserted into the buccal cavity were determined (Fig. 4). The most posterior-starting path from the nine observations (path 1 in Fig. 4B) showed a considerable anterior movement until shortly after the instant of peak jaw protrusion. Next, the sphere moved a short distance back towards the posterior side of the head, but the net displacement was 20% of head length in the anterior direction. A consecutive act of churning (path 2 in Fig. 4B) brought the sphere a similar distance forward, after which it moved ventrally towards the floor of the buccal cavity at the level of the hyoid (Movie 3 shows these two churning acts). Subsequently, there were a series of paths that moved with the depression and elevation of the hyoid (paths 3–9; Fig. 4B), with the exception of one (path 6; Fig. 4B) where the sphere was moved anteriodorsally up to the mouth. No movement of the sphere could be observed during the respiration cycles in between churning.

### DISCUSSION

Our analysis confirmed each of the hypotheses put forward based on the qualitative descriptions in the literature (Abraham, 1901; Oppenheimer and Barlow, 1968). Our X-ray videos provided the first quantitative evidence for forward movement of small objects inside the mouth during churning (Fig. 4). This confirmed the

description by Abraham (1901) for *P. philander* in our model species *O. niloticus*. A posterior-to-anterior wave of buccopharyngeal expansion, hypothesised to be necessary to cause such a flow, was indeed present during churning (Fig. 2). This expansion wave involved abduction of the opercula, abduction of the suspensoria, depression of the mouth floor by the hyoid and protrusion of the jaws. Also as hypothesised (see Introduction), abduction of the opercula indeed resulted in a considerable inflow of water entering the opercular slits (Fig. 3).

Together, these results suggest that the role of churning is more than just a mixing of the eggs to reposition those deprived of sufficiently oxygenating flows. The anterior flow of water entering through the gills will continue to move forward along with the expansion wave towards the front of the buccal cavity during churning. We hypothesise that this flow is generated to unblock the path of respiratory flow into the gills by moving the brood that may gradually become tightly packed by the posterior flows during respiration, or to prevent such obstructions from forming. This was also proposed by Abraham (1901), who noted that this gave the fish ‘an opportunity of relieving himself from the choking feeling he must have been constantly subjected to’. Because wriggling brood may less easily form a blockage near the gill entrance, this may also explain the decrease in the frequency of churning once the young hatch and become more mobile (Oppenheimer and Barlow, 1968). Additionally, as a slightly increased amplitude of the respiration cycles can cause a large number of the eggs to have a constant spiralling motion (except perhaps the most posterior ones, but this



**Fig. 4.** Path of a small sphere containing a radio-opaque marker during churning as determined based on nine high-speed X-ray videos. (A) The head-bound frame of reference and the monitored region. (B) Path lines for each churning sequence, coloured and numbered. The direction of movement and the position of the sphere at three instances are indicated by arrows (red arrow, 40 ms before the time of peak protrusion; black arrow, at the time of peak protrusion; green arrow, 40 ms after the time of peak protrusion).

cannot be observed), the need for churning only for this reason (as proposed by Oppenheimer and Barlow, 1968) seems unlikely. Such motion of the eggs during highly active respiration was observed when looking into the temporarily opened mouth (Oppenheimer and Barlow, 1968), which was also the case for the Nile tilapia during the present study.

An interesting finding is that the position along the gill slit of the inlet and outlet of water during churning is not the same. Flow enters into the opercular cavity at the dorsal part of the gill slit near the abducted opercula, but exits more ventrally where the branchiostegal membranes are connected to the hyoid (Fig. 3). This can be explained by the opercular region of the gill slit being opened actively (i.e. by force from the dilator operculi and levator arcus palatini muscles; Anker, 1978) and closed actively (i.e. by force of the adductor opercula and the adductor arcus palatini muscles; Anker, 1978), while the ventral region acts purely as a passive valve. Suction will be created during the opercular abduction phase, resulting in a lower water pressure inside compared with outside the head. Such a pressure gradient will automatically close the ventral branchiostegal valve. When afterwards the head is compressing and pressure becomes higher inside than outside the head, the water pressure will push the passive valve open but water cannot exit more dorsally on the gill slit because the opercula are firmly adducted at this instant. As a result, the intra-oral flow during churning will not be mirrored during the phases of anterior versus posterior flow, which may be important to guarantee a net forward displacement of the brood.

It cannot be excluded that churning also contributes to respiration of the mouthbrooder. As the posterior-to-anterior expansion wave continued up to the protruding mouth region (Fig. 2), and forward displacement of the eggs was observed (Fig. 4), it is likely that most of the water entering the opercular cavity will pass the gill lamellae and flow into the buccal cavity. Respiration through an opercular inflow of water has been observed in other fishes, generally in species that live at the bottom and have a ventral mouth. For example, sturgeons (*Acipenser transmontanus*) for which inflow through the mouth was experimentally eliminated were able to draw water into their branchial cavities through openings in the upper regions of the opercular slits (Burggren, 1978). Astroblepid catfishes have freed their suckermouth (used during climbing) from its inhalatory function by a duplication of the gill openings: only inflow through the incumbent gill openings was observed (De Crop et al., 2013). However, because of the relatively high impulse given to the water during churning in *O. niloticus* (higher amplitude of the expansion wave compared with respiration; Fig. 2), shunting of water around the gills is possible (Strother, 2013a,b). Consequently, it is not clear whether the flows generated during churning are useful for the gills of the parent fish in terms of extraction of oxygen. Despite the fact that the generation of such high-impulse flows is expected to result in a considerable energetic cost to the mouthbrooder, an oxygen consumption study of the cichlid *Pseudocrenilabrus multicolour* showed that the energetic cost of mouthbrooding is limited to an increase of only a few per cent (Mrowka and Schierwater, 1988).

The posterior-to-anterior wave of cranial expansion during churning will require a different motor programme (i.e. sequence of muscle activation) from the more general anterior-to-posterior waves of expansion that are much more commonly observed, for example, during respiration or suction feeding. This raises the question of the origin of such a reversed activation sequence; namely, whether this neuromotor pattern is a novel trait that appeared at the origin of mouthbrooding. This seems unlikely as

other actions that are more common in fishes share notable similarities. As mentioned in the Introduction, coughing is regarded as a normal part of the respiratory activity of most fish (Hughes and Adeney, 1977; Summers and Ferry-Graham, 2001), and often these are ‘forward coughs’ (Kuiper, 1907). Oral transport of offspring between excavated pits by substrate guarders is assumed to have provided the first step towards the evolution of mouthbrooding (McConnell, 1959; Goodwin et al., 1998). Forward coughs may be used to release the offspring after transport. However, the mouth is not closed in such coughs. This is a significant difference from the closed-mouth protrusion during churning. During intra-oral manipulation of food, however, we do see closed-mouth protrusions that appear remarkably similar to churning. The kinematics of closed-mouth protrusion acts during food processing have been described in detail in cyprinid fish (Callan and Sanderson, 2003; Gidmark et al., 2012). Yet, it remains to be shown whether the cranial kinematics of these actions during intra-oral food manipulation are identical to churning. Nevertheless, it is likely that the ability to perform actions analogous to churning preceded the evolution of mouthbrooding.

The functional morphology of posterior flow-driven transport of food inside the mouth cavity by aquatic fishes has been studied intensively during the past decades (Day et al., 2015). Consequently, the sequence of motion of the cranial elements involved during this typical transport of food towards the oesophagus entrance is well known. In contrast, relatively few studies have dealt with other types of intra-oral, hydrodynamic manipulation of items inside the mouth cavity (e.g. Liem, 1979; Drucker and Jensen, 1991; Konow and Sanford, 2008; Gidmark et al., 2012). How fish manage to separate food from debris, or position a bolus in between their pharyngeal jaws by hydrodynamic actions remains largely unknown (but see Drucker and Jensen, 1991). Such behaviours, together with the common capacity of fish to spit out undesirable items, and the churning kinematics described in the present study, illustrate the versatility of the buccal apparatus of fishes beyond generating the typical anterior-to-posterior flows during suction feeding. As argued above, this versatility might have paved the way for the evolution of mouthbrooding in cichlids.

In conclusion, our kinematical analysis has provided new insight into the role of churning during mouthbrooding, and how churning is performed by the cranial musculoskeletal system in the Nile tilapia. This species alternates between different motion sequences of its cranial elements when switching between respiration and churning during mouthbrooding. During churning, a posterior-to-anterior wave of cranial expansion and then compression is used instead of the more common, anterior-to-posterior wave that is used by fish for respiration and suction feeding. This reversed motion sequence reverses the direction of water flow: we observed an inflow of water through the opercular slits as well as a net anterior displacement of small objects initially located at the back of the mouth cavity. This anterior flow of water can help to prevent respiratory flow obstructions when dense accumulations of eggs are formed near the gills. Whether mixing of the eggs due to churning in combination with the inflow of fresh water from the back of the head is also important for the oxygen supply to the brood is a potential topic for future research.

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#### Competing interests

The authors declare no competing or financial interests.



**Author contributions**

S.V.W., G.D.B., D.A. and P.A. designed the study. M.D. and H.J.L. made the dual-view videos. I.J. analysed the kinematics and performed the flow visualisation. S.V.W. performed the particle tracking, recorded and analysed the X-ray videos, and wrote the manuscript; all authors discussed the results and commented on the manuscript.

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**Supplementary information**

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