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COMPLIMENTARY/POSTER SESSION PAPER

# Functional Morphology of the Urohyal Shunt for Symmetrical and Asymmetrical Ventilation in the Flatfish, *Isopsetta isolepis*

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**Synopsis** Flatfishes are benthic fishes that are well known for their ability to bury in the sediment, making the transition from above to below the sediment in a matter of seconds. Laterally flattened bodies allow flatfishes to lay flush against the substrate, a behavior facilitated by having an asymmetrical neurocranium with two eyes on one side of the head. Despite neurocranial asymmetry, their gill chambers are highly symmetrical. Additionally, most flatfishes have a uniquely shaped urohyal bone that forms passageway for water to travel ventrally between the “eyed-side” and “blind-side” gill chambers. Our study examines whether the kinematics and pressures generated by the gill chambers are also symmetrical during breathing above and below the sediment and during rapid burial in sediment. We studied *Isopsetta isolepis* individuals using sonomicrometry crystals to measure the changes in positions of the opercle bones relative to the urohyal and pressure transducers to record gill chamber pressures during burial. We conclude *I. isolepis* exhibit both symmetrical and asymmetrical breathing above and below the sediment. Pressures and movements were highly asymmetrical during burial jetting. We observed motions that indicate that the urohyal is an active shunt to allow passage of water between the eyed to the blind-side gill chambers.

## Introduction

Flatfishes (Pleuronectiforms) are benthic ray-finned fishes (Actinopterygii) primarily characterized by the evolution of several unique morphological adaptations, including their flattened bodies and asymmetrical neurocranium. Flatfish larvae hatch with a symmetrical head, and after several weeks, one of the eyes migrates to the “eyed side,” leaving the other side, the “blind side,” without eyes. (Friedman 2008; Schrieber 2013). As a result, flatfishes have two sides with differing functions. Adult flatfishes spend most of their time lying with the blind-side flush against the substrate. The eyed side faces upward, towards the water column, and solely the eyed side has adapted colors and patterns for camouflaging in their environment (Spinner et al. 2016).

The flatfish neurocranium has asymmetrical features, but the branchiostegal rays, opercular bones and gill openings remain anatomically symmetrical. Thus, flatfishes have a functional operculum and opercular valve on both sides of the body (Brainerd et al. 1997), like other actinopterygians. Additionally, flatfishes ventilate their gills using the same general pumping mechanism as all other ray-finned fishes (Hughes 1960; Liem et al. 1985). Ray-finned fishes have a mouth (buccal) chamber and left and right symmetrical gill chambers. To take in water for ventilation, they begin by expanding the buccal chamber so that water enters through the oral valve. While buccal expansion is occurring, ray-finned fishes begin to expand the gill chambers, drawing water across the gills. The buccal chamber then begins to compress, pushing the remaining water from the buccal chamber across the gills and into the gill chambers. Finally, ray-finned fishes force water out of the left and right gill openings by compressing the gill chambers (Farina and Bemis 2016).; Brainerd and Ferry-Graham 2005; Hughes 1960). This complex process is facilitated by the many cranial muscles that control the mouth, hyoid, opercular bones, branchiostegals, and gill arches.

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For example, hyoid retraction by the sternohyoideus muscle expands the mouth to draw in water in the initial expansive phase. Not much is known about the ecological and biomechanical diversity of aquatic pumping across ray-finned fishes, but it is generally accepted that, in benthic fishes such as flatfishes and other bottom-associated species, gill chamber expansion dominates the ventilation cycle (Farina and Bemis 2016; Long and Farina 2019; Hughes 1960).

Due to their unusual anatomy, ventilation in flatfishes has long been a topic of interest. Researchers have pointed out that symmetrical gill chambers present mechanical challenges for flatfishes, given their benthic orientation on the substrate, obstructing the movements of the blind-side operculum (Orcutt 1950; Yazdani and Alexander 1967). Asymmetrical gill chamber movements have several advantages for flatfishes. If flatfishes exhale out of the blind-side gill opening, they produce a jet of water that pushes them off the substrate, which is useful for fast starts off the bottom (Brainerd et al. 1997). If flatfishes exhale out of the eyed-side gill opening, they would be better suited to maintain adequate ventilation while remaining stationary on the substrate and minimally disturbing the surrounding sediment. However, the possibility of asymmetrical movements in flatfish ventilation has long been controversial. Hughes (1960) recorded pressure data from the left and right gill chambers of various pelagic and benthic fishes, which reportedly were highly symmetrical, but he noted observing possible asymmetrical movements in resting, non-instrumented flatfishes, such as plaice (*Pleuronectes platessa*) and merry sole (*Microstomus kitt*). Yazdani and Alexander (1967) study of species of the Bothidae, Pleuronectidae, and Soleidae families reported observing asymmetrical breathing in a flatfish, noting that water infused with dye was directed out of the eyed-side gill chamber only. However, Liem and colleagues (1985) strongly refuted these observations in their cineradiographic and pressure study of *Psuedopleuronectes americanus,* in which they observed only symmetrical movements and pressures. They went as far as to state that their experiments "have revealed unequivocally that the respiratory current and underlying mechanisms [in flatfishes] are symmetrical." A goal of our study is to resolve this controversy by documenting asymmetrical and symmetrical movements and pressures of flatfish gill chambers. One hypothesis for the conflicting past studies is that flatfishes exhibit both symmetrical and asymmetrical breathing. Digital recording technology now allows us to record movements over long periods of time, providing us with a more complete picture of the range of behaviors an individual can exhibit. It is also possible that asymmetry is species-specific, and so, therefore we will study a new species that has not been previously examined. Additionally, we provide conclusive evidence of urohyal motion during breathing. We are also the first to document ventilatory pressures during burial. While (Brainerd et al. 1997) inferred from video that flatfish can forcefully eject water from the blind gill opening during fast-starts from the substrate, we now directly measure pressure in both gill chambers during another rapid behavior: burial under the sediment.

Most flatfishes (with the exception of *Psettodes* and Cynoglossidae) have a highly specialized urohyal bone adapted for asymmetrical ventilation (Yazdani and Prakash 1990). In actinopterygians, the urohyal is an ossified tendon of the sternohyoideous muscle, which attaches anteriorly to the bones of the hyoid arch. The sternohyoideus is an important suction feeding and ventilation muscle that retracts the hyoid to expand the buccal chamber, and anatomically, it provides a barrier between the left and right gill chambers. In most flatfishes, the urohyal is suspended in the middle of the sternohyoideus and has a unique V-shaped curve that opens a channel between the eyed and blind-side gill chambers (Yazdani and Alexander 1967; Liem et al. 1985; Yazdani and Prakash 1990; Fig. 1). Yazdani and Prakash (1990) describe the Pleuronectidae urohyal as having a "hook-shaped or V-shaped" curved vertical plate with a posterior edge "bent down and turned forward," a ventral edge that forms "the cavity of the V" and a dorsal edge that sits under the gill arches and in front of the heart. Similarly, Liem et al. (1985) characterized the urohyal as having "a deep notch in the anterior edge" that forms an interconnecting channel between the eyed- and blind-side gill chambers that "can be easily demonstrated with a probe on both dead and living specimens." The positioning of the urohyal is shown in a schematic of *Isopsetta isolepis* in Fig. 1.

We propose that the urohyal allows for the movement of water between the eyed-side and the blind-side gill chambers when there is an asymmetrical activity in their gill chmabers, such that water pumped through the gills of the blind side passes through the urohyal channel to escape via the opercular opening of the eyed side when the fish is at rest on the sediment. The goal of this project was to determine the functionality of this urohyal canal and investigate the mechanisms by which flatfishes, specifically *I. isolepis* (butter sole), move water through their gill chambers and the urohyal channel using symmetrical or asymmetrical opercular movements during breathing and burial. We conducted experiments with *I. isolepis* individuals using surgically implanted sonomicrometry crystals and pressure transducers. We observed kinematics of ventilation during breathing above the sediment, below the sediment and during the burial event. We also document gill chamber during burial sequences and pressures to demonstrate opercular jetting.

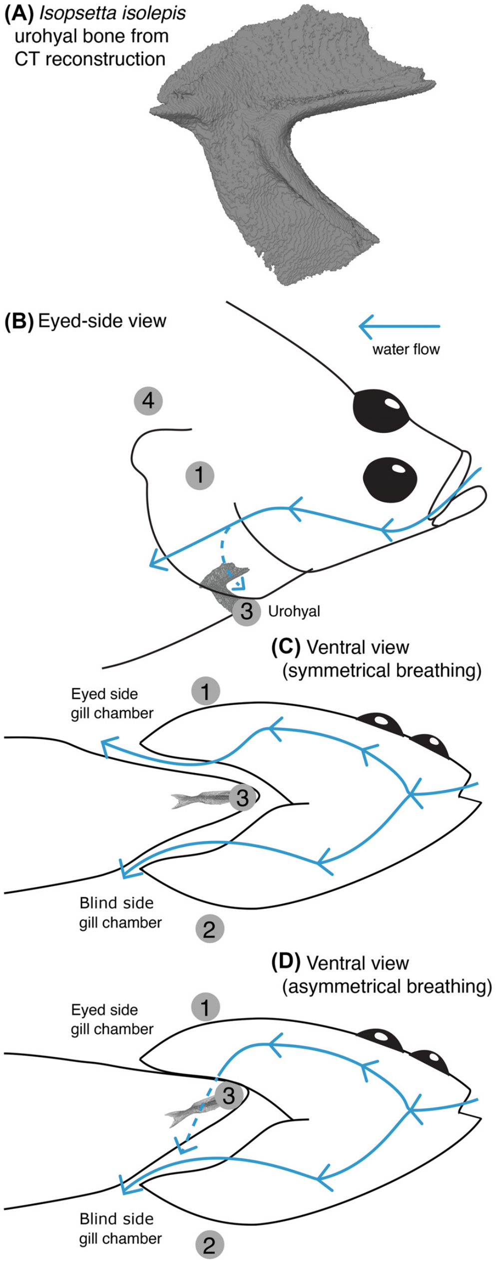

**Fig. 1** Flatfish gill chamber morphology and water flow. In *I. isolepis* and many other flatfish species, the urohyal sits between the eyed-side and blind-side gill chamber. A 3D model from a micro-CT scan of an *I. isolepis* urohyal (**A**) was added to a cartoon of an *I. isolepis* head (**B**), in its anatomical orientation, internal to the opercular bones forming the isthmus of the head (**C**). Sonomicrometry crystals were sutured to the skin on the eyed-side opercle (1), blind-side opercle (2), and the ventral tip of the urohyal (3). An additional crystal (4) was added as a reference point on the body, sutured on the skin above the epaxial musculature, as close to crystal (1) as possible. In flatfishes, water that enters the gill chambers from the pharynx can be directed out of eyed-side and blind-side gill openings during symmetrical breathing (**C**) or shunted to the opposite gill chamber during asymmetrical breathing (**D**) through a unique urohyal shunt (3). Arrows indicate water flow. Numbers indicate sonomicrometry crystal placements.

## Methods and materials

### Animal collection and care

We collected *I. isolepis* (butter sole) ($n = 5$) during beach seines at Jackson Beach (48°31′09.8″N, 123°00′37.4″W) and benthic trawls in the San Juan Islands near the Friday Harbor Laboratories in Friday Harbor, Washington, USA. The fish were housed in size-dependent groups in flow-through tanks and fed a diet of mysid shrimps or frozen prawn tails approximately once every 2–3 days. Protocols for animal husbandry were completed in accordance with the University of Washington IACUC approved protocol #4238–03. The fish were observed within four weeks of collection. All individuals were euthanized at the end of the study.

### Experimental conditions

We performed our experiments in seawater ranging from 13 to 15°C in an observation tank (24.5 × 12.5 × 16.75 inches) and filled with ∼1.5 inches of fine sand retrieved from a sandy beach. To determine the movements of the eyed-side and blind-side operculum bones, 2-mm-diameter piezoelectric sonomicrometry crystals were sutured onto the loose skin covering structures of *I. isolepis* individuals ($n = 4$) anesthetized with MS-222 (75 mg/L) in seawater buffered with an equal concentration of sodium bicarbonate. Sonomicrometry is a technique for measuring changes in the relative distance in mm between two crystals using acoustic signaling. Positions of the crystals were as follows: eyed-side opercle, blind-side opercle, urohyal, and the trunk (Fig. 1). The trunk crystal was sutured to the skin covering the epaxial muscles on the eyed side, as close to the eyed-side opercle crystal as possible. This trunk crystal served as a stationary point on the body for comparison with the crystals on the head. Individuals ranged in size from 15 to 20 cm in length and were observed for approximately an hour each to allow for the fish to complete several cycles of alternating ventilation and burial following a resting period of ∼10–20 min after recovery from anesthesia. We analyzed a total of 75 breaths from four individuals. At the conclusion of data collection, individuals were euthanized in MS-222 (250 mg/L) in seawater buffered with an equal concentration of sodium bicarbonate. Sutured crystals were removed after death was confirmed.

Eyed- and blind-side gill chamber pressures were recorded from a fifth individual (17 cm) from the eyed and blind gill chambers. Cannulae made from polyethylene tubing (PE 160) were surgically implanted through the opercle on both the eyed and blind sides in a fish

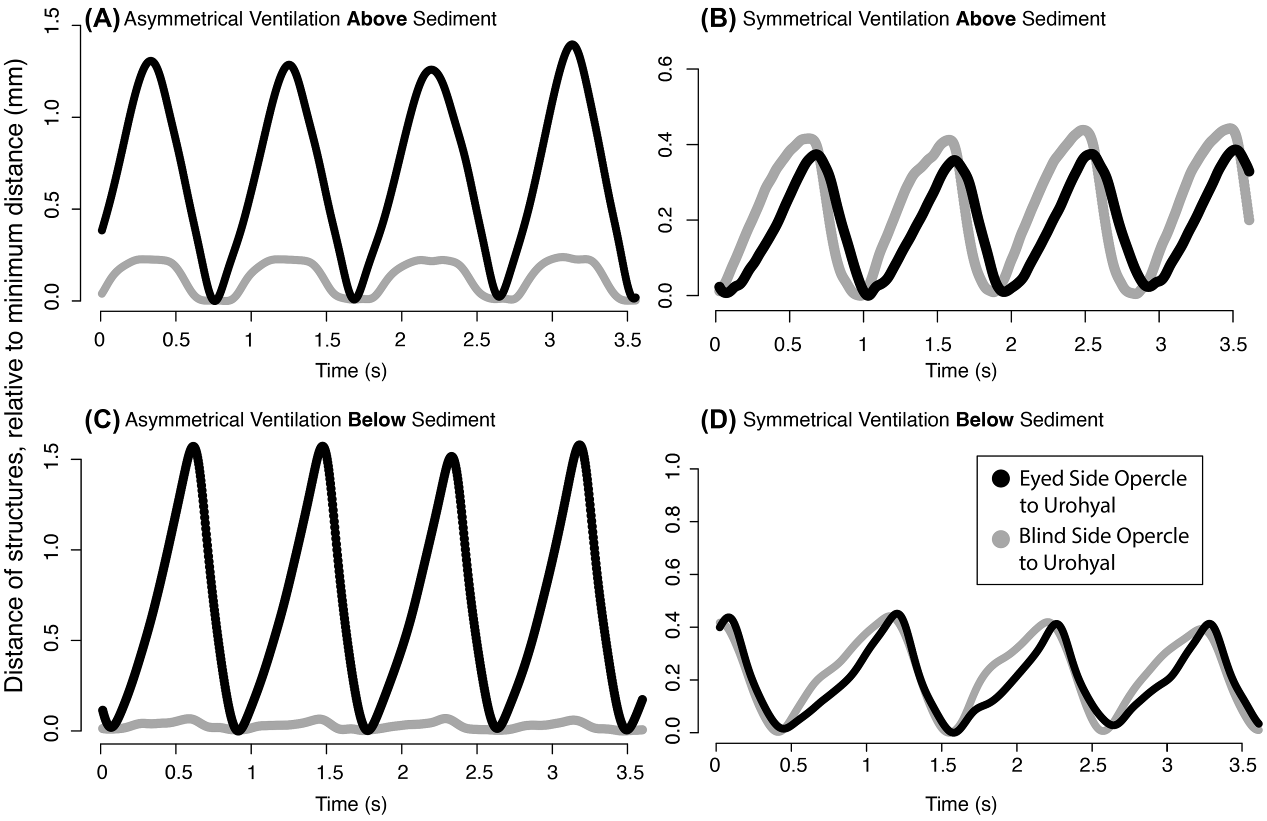

**Fig. 2** Example sonomicrometry crystal distance traces. We observed asymmetrical and symmetrical ventilatory kinematics above (prior to burial) and below (after burial) sediment. The asymmetrical sequences on the left (**A** and **C**) are from one individual, and the symmetrical sequences on the right (**B** and **D**) are from two other individuals. All individuals were observed performing all four behaviors.

anesthetized with MS-222 (75 mg/L). The internal end of the cannulae were flared to sit flush against the opercle. A Millar Mikro-Tip SPR-524 pressure transducer (Millar Houston, Texas, USA) was inserted into each cannula and allowed to enter the gill chamber slightly (∼0.5 mm). An additional pressure transducer recorded ambient pressure outside the eyed-side gill chamber in an external cannula paired with the eyed-side gill chamber cannula. Transducers were connected to a Millar PCU-2000 Dual Channel Pressure Control Unit (Millar Houston, Texas, USA), which was then wired to a LabJack T7 pro (LabJack Corporation, Lakewood, Colorado, USA) which is a data acquisition (DAQ) system controlled by LabJack software. Recording frequency was 30 Hz and pressure transducers were calibrated using two-point calibration with the control unit standards.

We used a Phantom camera to capture high-speed video of the ventral side of the head at a rate of 150 frames per second (fps). Movement of the urohyal in one individual (Fig. 3, bottom panel) was digitized from a high-speed video that we downsampled from 150 to 15 fps (Supplementary Video S1) for manual digitizing. We digitized the video using the StereoMorph package in R (Olsen and Westneat 2015) by manually tracking the tip of the urohyal. The video is a ventral view in which the urohyal can be seen moving laterally towards the eyed side during each breath. We also synchronized pressure for one burial event (Supplementary Video S2) using the Logger Pro 3 software so that we could annotate the behaviors (Fig. 6).

## Data analysis

For the sonomicrometry data, amplitudes of distance of the blind- and eyed-side opercle from the urohyal were obtained using a custom R script (R Core Team 2020) by which the minima and maxima for each breath were identified and subtracted to calculate amplitude. We adjusted the transmission frequency before and during data collection until there were no artifacts on the crystal distance data. We shifted the raw distance recordings so that minimum distance was set to zero to standardize the traces across figures. The crystal distance used for eyed-side gill chamber amplitude was between the eyed-side opercle and the urohyal, and the crystal distance used for blind-side gill chamber amplitude was between the blind-side opercle and the urohyal. The urohyal, positioned between the two gill chambers, was the middle reference point for amplitude cal-

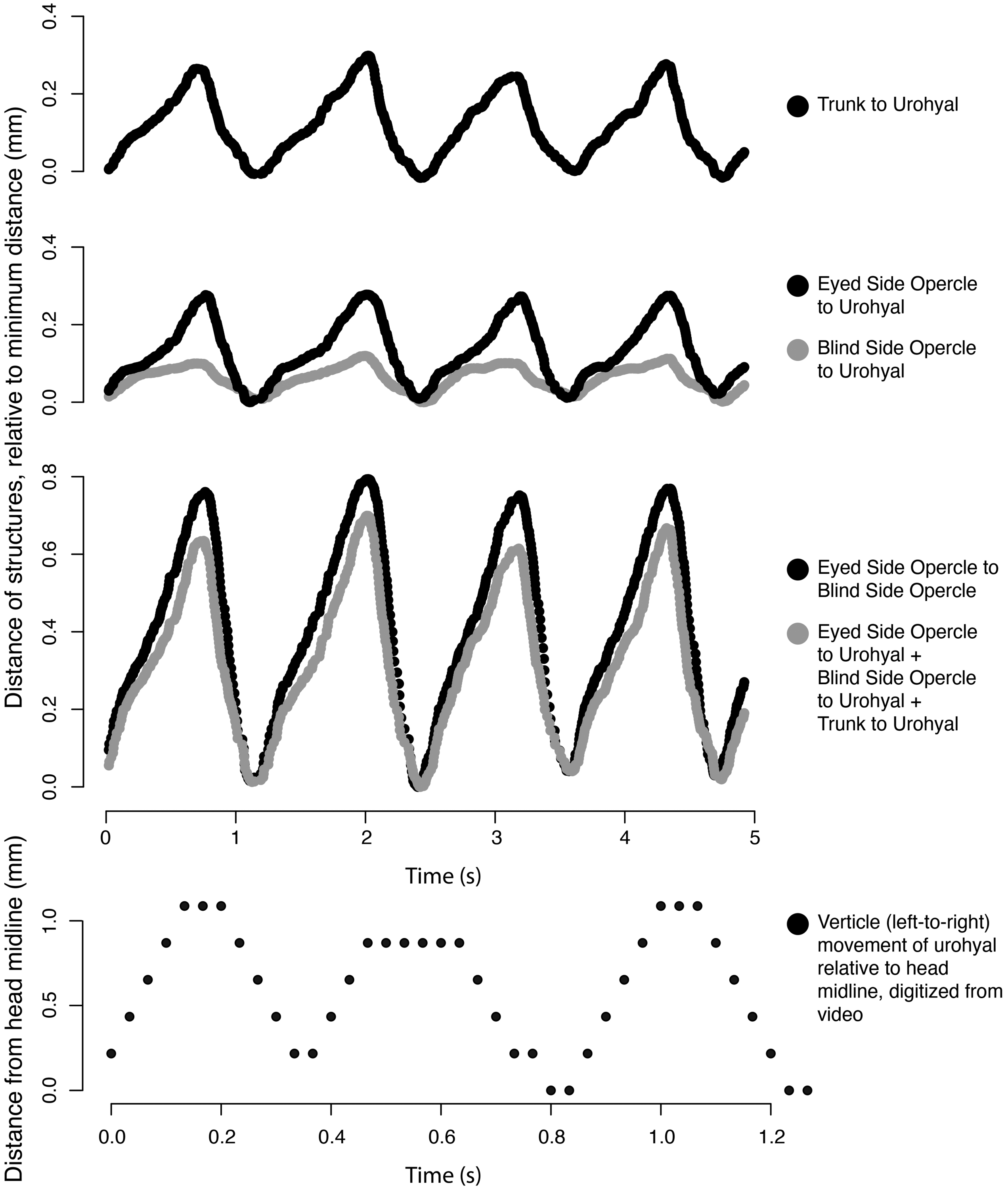

**Fig. 3** Kinematics of the urohyal and opercles during asymmetrical breathing. We document lateral movement of the urohyal (top) relative to the eyed-side trunk in one individual across four breaths. This movement greatly influences the asymmetry of the crystal measurements between the eyed side and urohyal and the blind side and urohyal (middle). With each breath, the urohyal makes a lateral excursion towards the blind side. Adding all of the following crystal movements (gray) gave a similar trace to this overall distance measurement: eyed opercle to urohyal + blind opercle to urohyal + trunk to urohyal. Additional evidence of lateral urohyal movements can be seen in a kinematic trace (bottom) from a video of another individual.

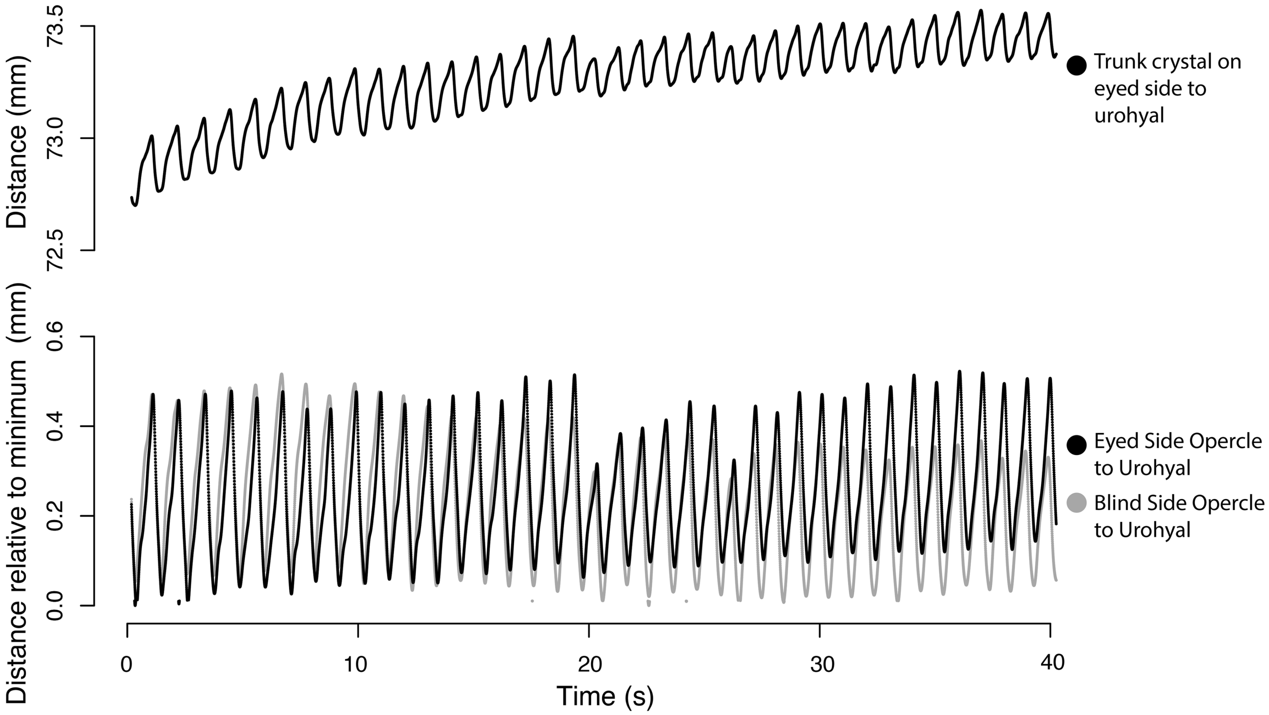

**Fig. 4** Sonomicrometry crystal distance traces as a fish changes head position. This sequence is from an individual who had just buried under the sediment. The urohyal started in a midline position between the eyed- and blind-side gill chambers. Over time, the urohyal repositioned to be farther away from the trunk crystal on the eyed side of the body (top) and closer to the blind-side opercle. This caused a shift from symmetrical opercle movements, relative to the urohyal, to asymmetrical movements (bottom).

culations. Because the urohyal functionally divides the left and right chambers and is considered the midline of the gill chambers in most other ray-finned fish, we considered it the best possible midline, even when it moved laterally. If any asymmetry in amplitude was visually observed in plots of sonomicrometry traces, we categorized that breathing as "asymmetrical." These amplitudes of 75 breaths across four individuals were compared using a two-factor ANOVA (Fig. 5) using the *aov* function in R (R Core Team 2020), with location (above or below sediment) and observed symmetry (symmetrical or asymmetrical) as the two independent variables.

We also made plots of filtered sonomicrometry data (Figs. 2–4) to demonstrate the differences in patterns seen in the ventilatory movements of these individuals. These data were filtered using a bidirectional, third-order Butterworth filter with a critical frequency of 0.1 using the *signal* package in R (R Core Team 2020; Signal 2014), and data were overlaid before and after filtering to ensure that there was no change in amplitude. We included a photograph to demonstrate buried ventilation (Fig. 7) and still images from high-speed video of a representative burial to demonstrate the use of opercular jetting during burial (Fig. 8). We also included a plot of pressure recordings from a burial event that was synchronized with the aforementioned video (Fig. 6), which provided the clearest and the least obstructed view of the burial sequence.

## Results

### Sonomicrometry crystals

We found that *I. isolepis* produce the following combinations of movements: asymmetrical ventilation above the sediment, symmetrical ventilation above the sediment, asymmetrical ventilation below the sediment, and symmetrical ventilation below the sediment. Sample sonomicrometry traces can be seen in Fig. 2. Two-factor ANOVA (Fig. 5) indicated that the breathing sequences that we categorized as asymmetrical were statistically significantly different from those categorized as symmetrical ($F = 94.08$, $P < 0.00001$), but there was no significant effect of location (above vs. below sediment; $F = 0.448$, $P = 0.505$). During burial behaviors, which included opercular jetting, we observed that eyed-side amplitude tended to exceed blind-side amplitude, with a mean blind-to-eyed side ratio of 0.583 (standard deviation of 0.575). Despite the higher amplitude pressure on the eyed side (Fig. 6), high-speed video revealed that water was primarily ejected from the blind side (Fig. 7), indicating that water was being shunted from the eyed gill chamber to the blind gill chamber to maximize jetting.

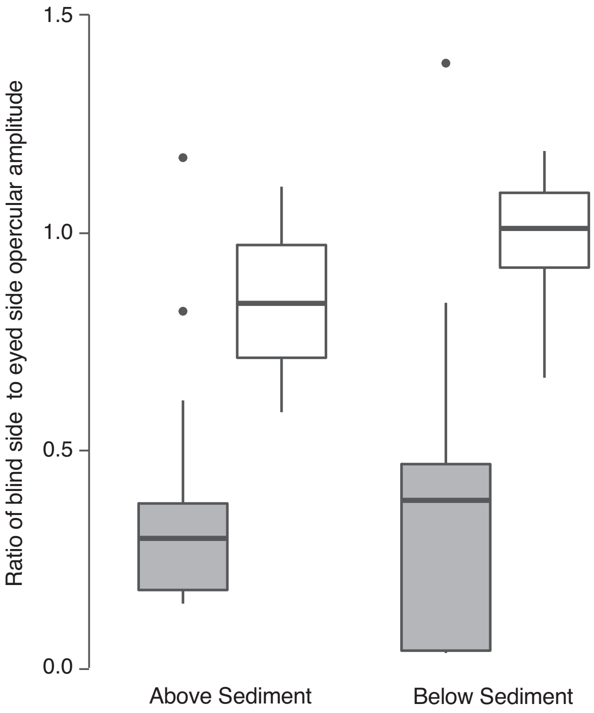

**Fig. 5** Comparison of amplitudes during different behaviors. Sonomicrometry data from 75 breaths were pooled from four individuals for statistical comparison. The ratio of blind-side opercle amplitude to eyed-side opercle amplitude was computed for each breath. A ratio of 1 indicated symmetry between the blind and eyed side, while a ratio below 1 indicated that the eyed-side amplitude was greater than the blind-side amplitude. A two-factor ANOVA showed significant differences between symmetrical and asymmetrical sequences, but there was no significant difference between buried breathing (below sediment) and above sediment breathing.

Sonomicrometry is a sensitive tool, especially when used to record crystals spaced relatively far apart (20–80 mm), which can produce artifacts. We controlled these factors to the greatest extent possible, making adjustments during the experiment to tune the crystals and check the data for precision. High-quality data above and below sediment ventilation were collected for each of the four individuals studied, for the eyed-side opercle, blind-side opercle, and urohyal crystals (Fig. 2). Because the trunk crystal was the furthest from the rest, quality recordings from this crystal were only obtained from one individual (see Figs. 3 and 4). We additionally observed a general trend of urohyal movement during ventilation ( Fig. 4). We commonly observed that each fish would adjust the positioning of the head over time. For example, post-burial, breathing would be symmetrical and the urohyal would be positioned directly between the eyed and blind-side gill chambers, aligned with the midline of the body. Over time, we observed a trend in which the urohyal would shift further away from the eyed side closer to the blind side (Fig. 4), to allow the blind-side opercle to sit flush against the substrate and for exhalation to be routed primarily through the eyed side.

Crystal distances can be difficult to interpret, because distance does not necessarily correspond to movement in a single anatomical plane. (Fig. 3) provides a detailed look into a full set of crystal data for a four-breath sequence from one individual (top three traces) and a kinematic tracking from a video of a different individual showing lateral urohyal movement. Here, we provide a description of the associated movements and how it informs our interpretation of the sonomicrometry results. Movements of the urohyal during breathing are lateral (Supplemetary Video S1) with a slight anterodorsal component. Therefore, distances between the trunk and urohyal crystals (in Fig. 3) largely represent lateral motion of the urohyal, although some anterodorsal motion is included. The lateral movements can be either towards the blind side (top three traces) or towards the the eyed side (bottom kinematic trace from a separate individual). The trunk was completely still during recordings. The “eyed-side opercle to blind-side opercle” distances represent the full amplitude of motion of abduction of the opercles. Opercular abduction and adduction were the only opercular motions associated with breathing, and we were careful to place the crystals in the same spot on the eyed and blind sides. We added “eyed-side opercle to urohyal,” “blind-side opercle to urohyal,” and “trunk to urohyal” to demonstrate that these distances, taken together, explain almost the entire amplitude and pattern of opercular abduction. We interpret this to mean that the urohyal in this trace moves laterally in the direction of the blind-side, explaining a substantial portion of the asymmetry in the opercular movements. The three graphs together show the complexity of this movement and illustrate that two types of asymmetrical movement are almost certainly occurring with each breath: lateral motion of the urohyal, and a lower amplitude of blind-side opercular abduction.

## Pressure transducers

We synchronized pressure transducer recordings with the high-speed video recordings from the Phantom camera to compare the pressure activity with the ventilation and burial activity of one individual. In Fig. 6, and 8 , the fish first produced symmetrical pressures during its light breathing phase above the sediment, then transitioned to a phase that included asymmetrical pressures caused by heavy breathing and coughing. Finally, the fish produced three large jets through large pressures of the eyed-side gill chamber (Fig. 6), which were shunted out the blind-side gill opening to

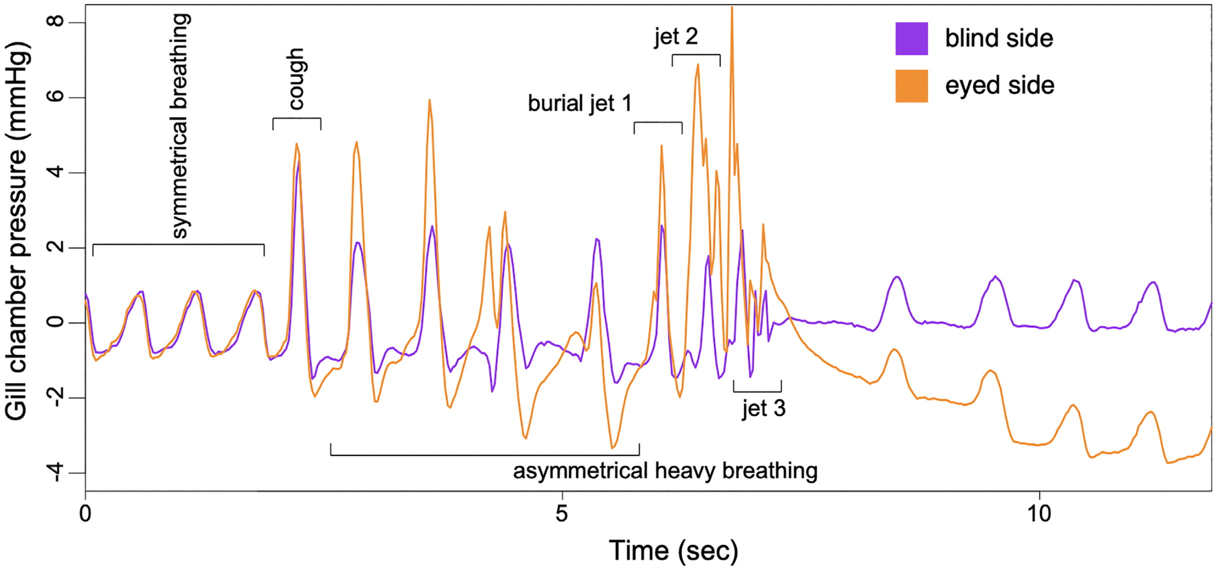

**Fig. 6** Ventilatory pressures during burial event. High-speed video and pressure recordings were synchronized to quantify ventilatory pressures during burial. This individual began with symmetrical breathing, followed by increasingly asymmetrical breathing, until the amplitude of pressure was higher in the blind side than the eyed side. During burial, the fish produced three substantial jets, presumably to fluidize the sand underneath the head (Fig. 8 B-D). Zero mmHg corresponds to ambient pressure, measured in a paired ambient transducer.

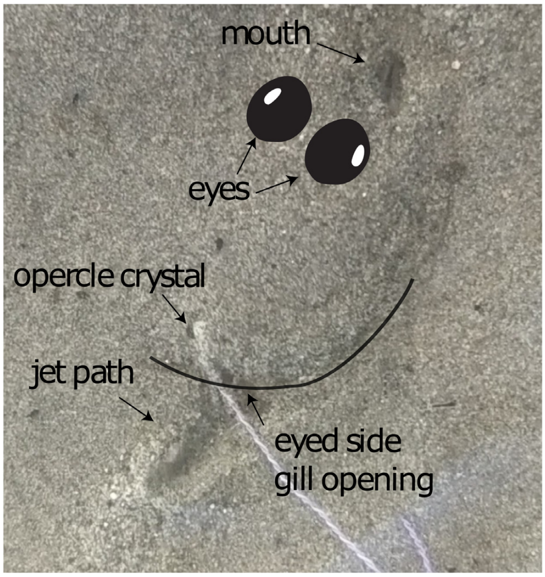

**Fig. 7** Image of eyed side breathing in buried I. isolepis. This image shows the typical condition of completely buried individuals in our experiment, with a small jet of water seen exiting the eyed side gill opening. The fish in the image above showed higher amplitudes of eyed side opercle movements relative to the blind side. The eyed side opercle boundary is indicated by the curved line.

displace plumes of sediment around the head (Fig. 7). This was followed by a momentary pause in opercular pressure before proceeding to breathe symmetrically below the sediment. Additionally, a still from a cellular phone video (Fig. 7) was included to show active jetting of the eyed-side gill chamber of a buried fish.

### Video records

Video recording of ventilation above the sediment captures one type of asymmetrical movement possible by flatfish during rest (Fig. 8A–D; Supplementary Video S1) and recordings of the fish during a burial event (Fig. 8E) show the kinematics of these movements as well as when sediment was displaced by jetting. The video stills in Fig. 8E (taken from Supplementary Video S2) capture the exaggerated compression and expansion of the eyed-side operculum during burial. Water leaving the blind-side gill chamber can be visualized in jets of displaced sediment (Fig. 8E).

All individuals were observed to have lateral movements of the urohyal during asymmetrical breathing. Video was challenging to obtain, due to the difficulty of getting orthogonal views from a fish on a sandy substrate without disturbing resting ventilation. We could also not obtain video of individuals buried under or obscured by the sand. Therefore, we confirmed lateral movement of the urohyal using a representative high-quality video (Supplementary Video S1). From the digitized video (Fig. 3, bottom panel), we see that the urohyal moves laterally towards the eyed-side opercle with each breath (Fig. 3, bottom panel). There is likely additionally a small amount of dorso-ventral rotation with each breath, which is why the total amplitude of the distance between the eyed-side trunk and the urohyal (Fig. 3, top panel) is greater than the lateral excursion of the urohyal observed in the digitized video (Supplementary Video S1).

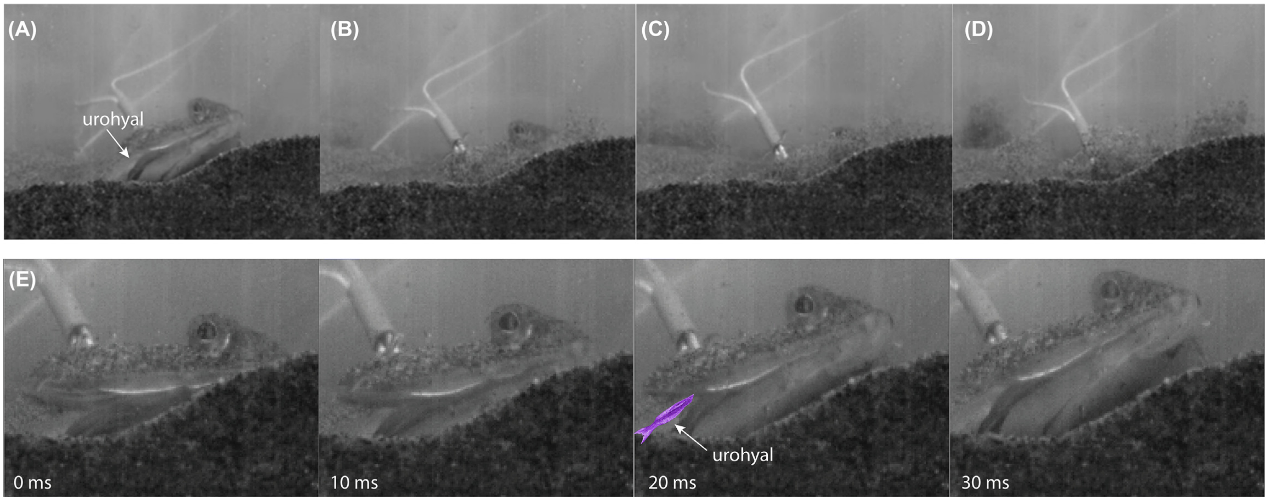

**Fig. 8** High speed video stills from a burial event. This video was synchronized with pressure (Fig. 6) to examine the roll of opercular jetting in burial. During heavy breathing prior to burial (see "asymmetric heavy breathing" in Fig. 6), exhalation is clearly seen occurring out of the blind side, despite higher pressures on the eyed side (Panel A). Panels B-D correspond to jets 1, 2 and 3, respectively in Fig. 6. Each jet produced a plume of sand around the head (visible in panels B-D), indicating the jets are exiting the blind side gill opening and fluidizing the sediment beneath the head. Panel E depicts the active lateral movement of the urohyal (from the midline at 0 ms to contacting the eyed side opercle at 30 ms) during pre-burial heavy breathing over 30 ms.

## Discussion

The symmetry of the gill chamber movements of *I. isolepis* changed dramatically throughout the recording periods (Fig. 2) in response to the positioning and behavior of the fish. Asymmetry in the sonomicrometry recordings can be explained by asymmetrical abduction of the eyed- and blind-side opercles, lateral movements of the urohyal, positioning of the urohyal, or a combination of these three. Asymmetry was measured as relative to the midline of the gill chamber, which is the urohyal. In ray-finned fishes, the sternohyoideus muscle and the urohyal are typically the structures that divide the left and right gill chambers ventrally in a region covered with skin called the "isthmus" (Farina et al. 2015). This anatomy is the same in flatfishes, except that in flatfish the urohyal forms a channel instead of a solid barrier. Additionally, the flatfishes urohyal is not connected to the hypohyal bones of the hyoid arch by ligaments and can therefore move relative to the hyoid, unlike in other ray-finned fishes. However, like other ray-finned fishes, the urohyal still forms the medial and ventral walls of the gill chambers. Thus, while the urohyal can change position, it is a midline structure that forms the dividing point for the eyed- and blind-side gill chambers, and we used it as a midline point for our symmetry measurements. We observed that *I. isolepis* tended to have symmetrical gill chamber movements when its head was propped up, leaving space for movement of both the eyed- and blind-side opercula. Orcutt (1950) also observed that the starry flounder (*Platichthys stellatus*) used its fins to prop the head above the sand, allowing symmetrical movements of the opercula. In above-sand breathing of propped up fish, we observed the urohyal directly in the middle of the adducting and abducting opercula, and this can be seen in the symmetry of the relative distances between the urohyal and either opercle (Fig. 2C). Over time, we observed that the fish would settle into a position with its head flattened against the substrate, at which point the eyed side would move with substantially higher amplitude than the blind side (Figs. 2A and 4). This dramatic change is likely due in part to asymmetrical abduction of the opercles. However, we noted that the urohyal would shift position as well, resulting in the ventilatory shunt to the eyed side after blind-side gill irrigation, for eyed side exhalation (Fig. 1D). Over the course of a single breath, the urohyal can also be observed to move laterally (Fig. 3; Supplementary Video S1). Therefore, the symmetry and direction of ventilatory flow is influenced by changes in movements of the opercles, position of the urohyal, and lateral movements of the urohyal.

The direction in which water leaves the gill cavities of flatfish (through either the eyed side or blind side) has long been a subject of debate in the literature. Hughes (1960) observed water leaving both gill chambers, using a glass-bottom tank to observe the blind-side gill openings. Yazdani and Alexander (1967) found that water primarily was routed out the eyed gill chamber when the fish was at rest. Liem et al. (1985)only observed symmetrical exhalation and questioned observations of asymmetrical exhalation by previous authors. Brainerd et al. (1997) observed opercular jetting out of the blind-side gill opening to break adhesion when performing a fast start off the substrate, although they did not comment on whether eyed-side exhalation was simultaneously observed. During resting ventilation, we observed symmetrical and asymmetrical movements of the opercles relative to the urohyal above and below the sediment (Fig. 2). During symmetrical movements, there is likely equal flow out both gill openings. However, asymmetrical breathing, when present during rest, involved large amplitudes of the eyed-side opercle, during which the eyed-side gill chamber likely had a much greater outflow than the blind-side gill chamber (Fig. 2A and C). In symmetrical, above-sediment breathing, we observed both gill chambers opening simultaneously, particularly when the fish were using their fins to lift their head so that the blind-side operculum was not resting against the sediment. Our study confirms that all these behaviors can be observed in *I. isolepis,* which likely explains the discrepancies of previous authors, who were limited by a lack of digital recording tools for making observations over multiple hours. Additionally, Liem et al. (1985) agreed that asymmetrical opercular movements, if observed, would indicate asymmetrical flow and passage of water through the urohyal. However, future studies of flow will be required to conclusively confirm that asymmetrical cranial movements are producing flow through the urohyal.

The uniquely shaped urohyal facilitates asymmetrical ventilation in flatfishes by providing an open connection between the two chambers between which water can flow. Sonomicrometry recordings measured movement of the urohyal relative to the trunk, showing that the urohyal could change position within a single breathing (Fig. 3) and over long periods of time as the fish adjusted position (Fig. 4). The mechanism for cyclical lateral movement of the urohyal during each breath is likely asymmetrical contraction of some or all of the sternohyoideus muscle and possibly asymmetrical contraction of some hypaxial muscle fibers associated with the pectoral girdle. The mechanism for repositioning of the urohyal over long periods (minutes) likely involves the cranial portions of the epaxial and hypaxial muscles, which are responsible for head positioning. Liem et al. (1985) noted anteriodorsal and posterioventral movements of the urohyal, but did not document lateral urohyal movement seen in our study. Lateral movement of the urohyal, which is typically the "midline" between the two opercula, calls into question the nature of asymmetrical movements when the midline frame of reference also continuously moves. This was our reasoning for adding a fourth crystal on the eyed-side trunk (Fig. 1), which allowed us to compare movements of the urohyal to a stationary reference point (Fig. 3). It is possible that the asymmetrical kinematics that we recorded were symmetrical with respect to the overall body midline, but not with respect the typical midline of the gill chambers (the urohyal and sternohyoideus). Liem et al. (1985) noted symmetrical electromyographical recordings from the levator operculi and dilator operculi, which could indicate that muscle activation patterns are symmetrical but the effects of the activity on gill chamber volume are not. Additionally, during burial, asymmetrical activity resulted in jets of water entering the sediment from the blind-side gill opening to displace sediment near the head. This jetting is facilitated by the urohyal shunt, allowing the passage water from the eyed to the blind side, although internal flow visualization would be necessary to demonstrate this more thoroughly.

## Conclusion

Our results explain discrepancies in prior characterizations of flatfish ventilation. The wide variety of behaviors that we observed in this species are consistent with most of the previous reported observations. Based on our physiological and biomechanical observations of *I. isolepis*, the following conclusions can be drawn: (1) This species can exhibit both symmetrical and asymmetrical movement of the operculum, with respect to the gill chamber midline, above and below the sediment. Asymmetry, when present, was exceptionally large in magnitude, with flatfishes "favoring" either the blind or eyed side, depending on how the head and gill chambers were positioned in the substrate. These different positions might be induced in the natural environment by abiotic structures, the presence of predators or other stressors, or whether the fish is actively hunting, preparing to ambush, or resting. (2) *Isopsetta isolepis* can shift from symmetrical to asymmetrical movement during ventilation while resting above or beneath the sediment. (3) *Isopsetta isolepis* can control the movement and activity of the urohyal and opercles during ventilation to inject jets of water into sediment to enhance fluidization of the sediment during burial. We posit the diverse behavioral repertoire present in this species, consistent with other observations in the literature, supports the conclusion that diverse systems of evolutionary adaptations are present not only in *I. isolepis,* but across many species within the clade. Subsequent ventilation studies will reveal the unique characteristics of the ventilation process through study of other Pleuronectiforms species and other marine organisms.

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## Supplementary data

Supplementary Data available at *ICB* online.

## Conflict of interest

We have no conflicts of interest to report.

## Data availability statement

Data are archived on the Open Science Framework portal (https://doi.org/10.17605/OSF.IO/UMD76).

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