# The mechanosensory lateral line is used to assess opponents and mediate aggressive behaviors during territorial interactions in an African cichlid fish

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## ABSTRACT

Fish must integrate information from multiple sensory systems to mediate adaptive behaviors. Visual, acoustic and chemosensory cues provide contextual information during social interactions, but the role of mechanosensory signals detected by the lateral line system during aggressive behaviors is unknown. The aim of this study was first to characterize the lateral line system of the African cichlid fish *Astatotilapia burtoni* and second to determine the role of mechanoreception during agonistic interactions. The *A. burtoni* lateral line system is similar to that of many other cichlid fishes, containing lines of superficial neuromasts on the head, trunk and caudal fin, and narrow canals. *Astatotilapia burtoni* males defend their territories from other males using aggressive behaviors that we classified as non-contact or contact. By chemically and physically ablating the lateral line system prior to forced territorial interactions, we showed that the lateral line system is necessary for mutual assessment of opponents and the use of non-contact fight behaviors. Our data suggest that the lateral line system facilitates the use of non-contact assessment and fight behaviors as a protective mechanism against physical damage. In addition to a role in prey detection, the diversity of lateral line morphology in cichlids may have also enabled the expansion of their social behavioral repertoire. To our knowledge, this is the first study to implicate the lateral line system as a mode of social communication necessary for assessment during agonistic interactions.

KEY WORDS: Aggression, Assessment, Mechanoreception, Neuromast, Social behavior, Teleost

## INTRODUCTION

Fish must integrate information from multiple sensory modalities to mediate adaptive social behaviors under varying environmental conditions and contexts. While many sensory systems are used during typical behavioral interactions in fishes, a disproportionate amount of research has focused on the role of visual (Chen and Fernald, 2011; Grosenick et al., 2007; Korzan and Fernald, 2007; Korzan et al., 2008; Rosenthal and Ryan, 2000), auditory (Amorim et al., 2003, 2004; Lobel, 1998; Maruska et al., 2012; Simões et al., 2008) and chemosensory signals (Almeida et al., 2005; Barata et al., 2007; Keller-Costa et al., 2015; Martinovic-Weigelt et al., 2012; Maruska and Fernald, 2012). In contrast, little is known about the role of the mechanosensory lateral line system in mediating adaptive social behaviors, especially during territorial or aggressive encounters.

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The mechanosensory lateral line system senses changes in the environment through detection of near-flow water movements relative to the fish (Coombs, 1994; Coombs et al., 1996; Dijkgraaf, 1962; McHenry and Liao, 2014). The functional unit of the lateral line system is a neuromast composed of sensory hair cells and support cells covered by a gelatinous cupula (Dijkgraaf, 1962). Neuromasts are located either superficially on the skin surface (superficial neuromasts, SN) or enclosed within dermal canals (canal neuromasts, CN) (Webb, 1989). Neuromasts are stimulated when the cupula is deflected by viscous drag, activating the mechanotransduction channels in the hair cells, and allowing for sensory perception of water movements relative to the movement of the fish (van Netten and McHenry, 2014). Lateral line system morphology is highly variable across fishes (Webb, 1989, 2014), and its spatial organization within a species can provide insight into its sensory role(s).

The lateral line system has been well studied for its involvement in topographic interactions with the environment (e.g. rheotaxis; Kulpa et al., 2015; Montgomery et al., 1997), sensation of water movements generated by other fish (e.g. schooling behavior; Pitcher et al., 1976; and predator–prey interactions; Coombs and Patton, 2009; Schwalbe et al., 2012), but few studies have examined the role of the lateral line system for sensing intentional water movements during intra-specific social communication. For example, a few studies have demonstrated that lateral line-mediated vibrational communication is used during reproductive and spawning behaviors in different fishes (Marchesan et al., 2000; Medina et al., 2013; Satou et al., 1994). In contrast, while a hypothesized role for the lateral line system during aggressive interactions has been suggested in the literature for years (Enquist et al., 1990), there is currently no direct experimental support for the role of mechanoreception in mediating territorial behaviors in any fish species. As aggressive displays typically include body and fin movements that generate hydrodynamic cues (e.g. chases, opercular displays, tail movements, body quivers; Keenleyside and Yamamoto, 1962; McMillan and Smith, 1974; Stewart et al., 2013; Stewart et al., 2014; Yoshizawa et al., 2014), the reception of these signals by the lateral line system is poised to provide crucial information for opponent assessment and to mediate appropriate behavioral decisions.

The African cichlid fish *Astatotilapia burtoni* is a highly social fish with well-characterized aggressive and courtship behaviors (Fernald, 1977; Fernald and Hirata, 1977; Maruska and Fernald, 2010b). Male *A. burtoni* exist as two distinct phenotypes (dominant and subordinate) and can reversibly switch between the two depending on their social environment, causing a suite of rapid behavioral and physiological changes (Maruska, 2014; Maruska and Fernald, 2014). Dominant, territorial males are brightly colored and aggressively defend a territory and court females. Subordinate, non-territorial males physically resemble females and are

| List of abbreviations | |
|---|---|
| BL | body length |
| CN | canal neuromast |
| DASPEI | 2-[4-(dimethylamino)styryl]-$N$-ethylpyridinium iodine |
| dpf | days post-fertilization |
| IO | infraorbital canal |
| $M_b$ | body mass |
| MD | mandibular canal |
| N | nares/naris |
| OT | otic canal |
| pLLn | posterior lateral line nerve |
| PO | postotic canal |
| PR | preopercular canal |
| SL | standard length |
| SN | superficial neuromast |
| SO | supraorbital canal |
| ST | supratemporal canal |
| T | trunk canal |

reproductively suppressed. Social status is tied to reproductive success, feeding opportunities and growth rate (Hofmann et al., 1999; Maruska and Fernald, 2014), and in *A. burtoni* is dependent on a male's ability to successfully defend his spawning territory. To do this, males use a variety of aggressive behaviors, such as lateral displays, border fights, biting and mouth fights (Fernald and Hirata, 1977; Table 1). During lateral displays and border fights, fish are in close proximity (<1 body length, BL), but not physically touching. While these behaviors produce visual cues, they also involve pushing water at each other, likely activating the lateral line system. Other behaviors, such as biting and mouth fighting, involve physical contact but also likely stimulate the lateral line system because of the close proximity of the opponent. In addition to territorial interactions, *A. burtoni* males produce hydrodynamic cues during courtship behaviors via body quivers, tail waggles and leads, presumably also providing relevant information (e.g. size, fitness) to potential mates. While visual, acoustic and chemosensory signaling is used during *A. burtoni* social interactions (Chen and Fernald, 2011; Maruska et al., 2012; Maruska and Fernald, 2012), it is also likely, but untested, that hydrodynamic information influences social decision making in this species.

The goals of this study were to first characterize the morphology and spatial distribution of the mechanosensory lateral line system in *A. burtoni*, and then to ablate the lateral line system to test the hypothesis that mechanoreception plays an important role in male–male territorial interactions.

## MATERIALS AND METHODS
### Experimental animals
Adult laboratory-bred *Astatotilapia burtoni* (Günther 1894) were derived from wild-caught stock from Lake Tanganyika, Africa, and maintained in an environment that mimicked their natural habitat. Fish were housed in 30 l aquaria at 28–30°C on a 12 h light:12 h dark cycle and fed cichlid flakes (AquaDine, Healdsburg, CA, USA) once daily, supplemented with brine shrimp. Animal care and experimental procedures followed approved Louisiana State University IACUC protocols.

### Characterization of the mechanosensory lateral line system
To characterize and determine the distribution of the canals and pores of the lateral line system in *A. burtoni*, a 0.1% Methylene Blue solution was injected into cranial canal pores of freshly killed fish, resulting in visible blue stain throughout all cranial canals (*N*=6). Trunk canals were visible without staining using a stereomicroscope (SteREO Lumar V.12, Zeiss, Germany). Two adult male *A. burtoni* [standard length (SL)

**Table 1. Definition and classification of male *Astatotilapia burtoni* aggressive behaviors**

| Behavior | Classification | Definition |
|---|---|---|
| Chase | Non-contact | One fish chases the other around the compartment |
| Lateral display | Non-contact | Fish is within 1 BL and either parallel or perpendicular to opponent with spread opercula, erect fins and distended chin |
| Fake bite/ mouthing | Non-contact | Fish perpendicular to opponent within ¼ of a body length and opening/closing mouth without physical contact |
| Lunge | Non-contact | Quick movement toward opponent without physical contact; mouth closed |
| Frontal threat | Non-contact | Slower movement towards opponent with spread opercula |
| Abnormal lateral display | Contact | Qualities of a normal lateral display, but performed with direct contact to opponent instead of at 1 BL distance |
| Mouth fight | Contact | Two individuals grasp one another by the mouth and push, pull, bite and turn |
| Bite | Contact | With mouth open, one fish makes physical contact with the other, typically on the body of the opponent |
| Nudge/ram | Contact | With mouth closed, one fish pushes the other |
| Following behavior | Non-fight | One fish follows the opponent within 1 BL |
| Approach and back down | Non-fight | Swimming quickly up to the opponent and then shying away |
| Within 1 BL | Non-fight | Fish within 1 BL of each other and not performing any other behavior |

Fight behaviors were classified as either non-contact [behavior performed within 1 body length (BL) of opponent but no physical contact] or contact (physical contact between fish). Non-fight behaviors occurred either prior to fight onset or during trials in which no fight occurred.

71.50±4.50 mm, mean±s.e.m.] were also cleared and stained for further visualization of cranial and trunk canals (procedures outlined in Taylor and Van Dyke, 1985). For visualization of SNs on the skin and CNs inside the canals, fish [6 females, 6 males, 4 juveniles at 12 days post-fertilization (dpf); *N*=16 total] were immersed in 0.008% DASPEI (2-[4-(dimethylamino)styryl]-*N*-ethylpyridinium iodine; Molecular Probes, Carlsbad, CA, USA) solution for 20 min, killed in 0.01% benzocaine (Sigma, St Louis, MO, USA) dissolved in cichlid-system water, and imaged using an eGFP filter set (excitation filter 485/20; 525 LP filter) on a stereomicroscope. Neuromasts were counted, classified by location and averaged for each sex, and a composite distribution map was created using the most common placement and number of neuromasts across all animals. Canals and canal pores were then superimposed on the drawing to collectively represent the entire mechanosensory system.

### Behavior experiments
To test the role of the lateral line system in aggressive interactions, fish were placed in a novel paradigm in which they had equal opportunity to acquire a territory. A single 10 gallon tank (50.8×31.12×25.4 cm) was divided into two equal compartments by a removable opaque acrylic barrier and a quarter of a terracotta pot was placed on either side of the barrier to serve as a territory for each subject fish (Fig. 1A). Experimental dominant male fish [SL 42.625±0.769 mm, body mass (*M*_b) 2.148±0.665 g, *N*=150 total] were chosen based on their displays of typical dominance behaviors in community tanks for ∼5 days before being moved to the experimental
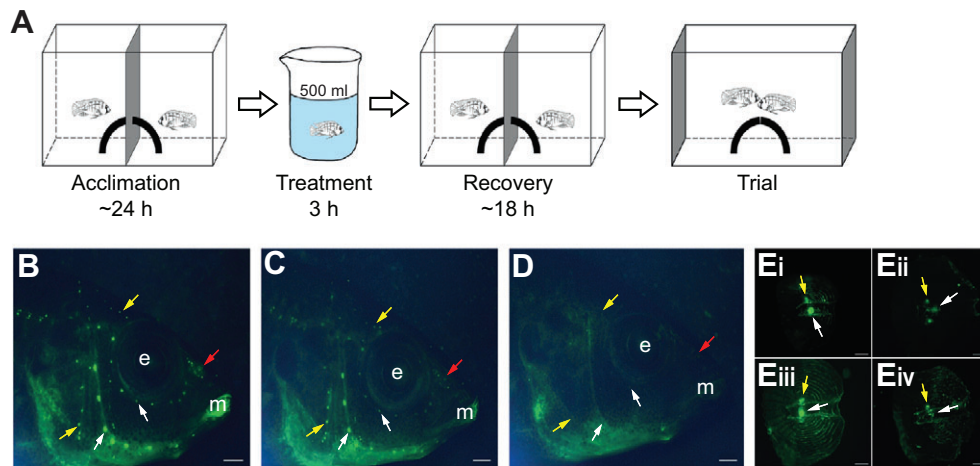
**Fig. 1. Experimental tank set-up for territorial interactions between dominant male *Astatotilapia burtoni* and verification of lateral line ablation treatments.** (A) Two dominant males acclimated on either side of an opaque barrier for 2 days with a quarter of a terracotta pot to serve as a territory. Fish were treated for 3 h (cobalt chloride or sham) and placed back in the experimental tank to recover for ~18 h prior to behavioral testing. On the day of the trial, the center barrier was removed and the two quarter pots repositioned to form a single half-pot territory to induce a territorial fight. Opaque barriers were placed on both ends of the tank to block the view of neighboring fish during trials. (B–E) DASPEI staining of neuromasts was used to verify treatment efficacy. Fish treated with 1 mmol l$^{-1}$ EGTA (B) and 0.1 mmol l$^{-1}$ CoCl$_2$ (C) had normal cranial neuromast staining (green dots) but fish treated with 2 mmol l$^{-1}$ CoCl$_2$ (D) had reduced or absent staining of cranial neuromasts. e, eye; m, mouth. Yellow, white and red arrows indicate canal neuromasts, superficial neuromasts and the naris, respectively. Arrows are in consistent locations in B–D, demonstrating the absence of staining at arrows in D. (E) None of the treatments affected trunk neuromasts: Ei, normal fish water; Eii, 1 mmol l$^{-1}$ EGTA; Eiii, 0.1 mmol l$^{-1}$ CoCl$_2$; Eiv, 2 mmol l$^{-1}$ CoCl$_2$. Scale bars: B–D, 1 cm; E, 200 μm.

tank to acclimate to their new territory for 2 days. Experimental fish were randomly assigned to one of three groups (control, lateral line ablation or sham handling; see below), resulting in four different groups of paired interactions: control fish versus control fish ($N$=12), sham fish versus sham fish ($N$=15), sham fish versus ablated fish ($N$=14), and ablated fish versus ablated fish ($N$=20). Each animal was used for only one behavior trial. Animals were size matched for both SL and $M_b$ so that no fish was more than 10% larger than his opponent, and to ensure that SL and $M_b$ did not differ across trial pairings (ANOVA, SL: $F_{2,53}$=0.969, $P$=0.386; $M_b$: $F_{2,53}$=0.427, $P$=0.654).

On the morning (08:00 h–08:30 h) of the trial, fish were allowed to acclimate to the video camera for ~10 min before a 5 min pre-trial behavior baseline was recorded. The opaque barrier was then removed and the two quarter pots were repositioned to form a single half-pot territory. In this paradigm, the two dominant males with individual territories are forced to fight over the new single shelter in the same tank and have equal opportunity to acquire the territory. During the trial, opaque barriers were also placed on either end of the tank to block the view of fish in adjacent tanks.

All trials were recorded and later quantified for pre-fight and fight behaviors, including contact (fish physically touching each other) and non-contact (fish within 1 BL but not physically touching) behaviors (Table 1). Fight onset was defined as the first reciprocal exchange of aggressive behaviors, and fights had to last a minimum of 30 s. The fight was allowed to continue until a clear winner and loser was established based on criteria similar to those used previously in a study on social defeat (Maruska et al., 2013). Specifically, the winner fish had to fulfill two of the following characteristics: (1) enter the shelter >3 times within a 1 min period, (2) enter and stay in the shelter for >10 consecutive seconds, (3) perform at least three dominance behaviors within 1 min, and (4) chase or bite the other male. The loser fish had to fulfill both of the following criteria: (1) loss of eye bar and bright coloration, and (2) perform typical submissive behaviors (e.g. fleeing, hiding). Latency to fight was defined as the time between removing the barrier and the fight onset, and fight duration as the time between fight onset and establishment of a winner. If no fight occurred within 30 min, the trial was ended and only non-fight behaviors (i.e. time with in 1 BL) were quantified, and a latency time of 30 min was assigned. If during the 30 min trial one fish became dominant and the other subordinate without an aggressive interaction occurring, the trial was marked as a 'no-contest win' and behavior data were not used.

### Ablation of the mechanosensory lateral line system

To compare behavior of lateral line-intact and lateral line-ablated fish, experimental fish were randomly assigned to one of three groups prior to use in the behavioral experiments described above: control, sham handled or lateral line ablated. Control fish were handled exactly as described above with no additional treatment. There are a variety of methods for pharmacological and chemical ablation of the lateral line system, such as treatment with aminoglycoside antibiotics or cobalt chloride (CoCl$_2$) (Brown et al., 2011; Janssen, 2000; Karlsen and Sand, 1987; Song et al., 1995; Van Trump et al., 2010). As pilot experiments showed that treatment with gentamicin in *A. burtoni* was only effective after a 4 day exposure at much higher than the recommended dose, we opted to use CoCl$_2$. We first tested 0.1 mmol l$^{-1}$ CoCl$_2$ for 24 h (as recommended by Janssen, 2000; Karlsen and Sand, 1987), but this treatment resulted in only slightly reduced DASPEI staining of cranial neuromasts and had no effect on trunk neuromasts. Through pilot experiments, we determined that a 3 h treatment with 2 mmol l$^{-1}$ CoCl$_2$ in low-calcium cichlid-system water effectively ablated >90% of the cranial lateral line system, but still had minimal effect on the trunk lateral line. This CoCl$_2$ treatment was therefore combined with severing the posterior lateral line nerve (pLLn), which resulted in near ablation of the entire lateral line system. Transecting the pLLn was shown to have similar behavioral effects to CoCl$_2$ and antibiotic treatment in other species (Mirjany et al., 2011). Lateral line ablation was done by immersing fish for 3 h in cichlid-system water containing 2 mmol l$^{-1}$ cobalt chloride hexahydrate (Sigma) with 1 mmol l$^{-1}$ EGTA (calcium chelating agent; Sigma) that was pH corrected to ~8.0 with NaOH (Fisher Scientific, Pittsburgh, PA, USA) after CoCl$_2$ and EGTA were dissolved in cichlid-system water. All solutions used in these experiments were adjusted in a similar manner so that pH was between 7.8 and 8.1. Immediately following CoCl$_2$ treatment, fish were placed in ice-cold cichlid-system water for 3 min before the pLLn was bilaterally transected. To cut the nerve, 2–3 scales were gently removed at the posterior dorsal edge of the operculum and a scalpel was used to make a small incision ~4 mm in length through the skin and superficial musculature. The pLLn was then visible and a 2 mm portion of the nerve was removed. CoCl$_2$ treatment caused ~90% ablation of cranial CNs and SNs (verified by reduced/absence of DASPEI staining; $N$=6), and transecting the pLLn removed both SN and CN input from the trunk and caudal fin.

Sham-handled fish were immersed in normal cichlid-system water for 3 h. Following treatment, fish were placed in ice-cold cichlid-system water and a small incision was made into their dorsal musculature near the dorsal

fin to mimic the surgery of pLLn-transected fish but ensuring that it did not affect the lateral line system. Following treatment or sham handling, fish were returned to the experimental tank overnight, and behavior experiments took place 18 h after the end of treatment, during which time neuromast function did not recover from $CoCl_2$ (verified by reduced/absence of DASPEI staining; $N=4$).

### Toxicity and anosmic controls

As a calcium channel antagonist, it is possible for cobalt chloride treatment to have unwanted effects, most notably toxicity effects leading to death (Janssen, 2000; Ridgway and Kamofsky, 2006). To ensure that any observed behavior differences were due to lack of mechanoreception, we included several toxicity and anosmic (elimination of olfactory inputs) controls. Previous research suggested 0.1 mmol $l^{-1}$ as the highest dose usable in fish (Janssen, 2000); however, a 2 mmol $l^{-1}$ $CoCl_2$ treatment was the lowest dose that consistently ablated the cranial portion of the *A. burtoni* lateral line system but still had little effect on trunk neuromasts. As this dose is much higher than recommended by Janssen (2000), we included several controls to ensure any behavioral changes were not due to toxicity of $CoCl_2$, EGTA or NaOH (used to raise the pH of the treatment solution to ∼8.0). Fish were treated for 3 h in either 2 mmol $l^{-1}$ $CoCl_2$ with no EGTA (pH adjusted with NaOH) or 1 mmol $l^{-1}$ EGTA with no $CoCl_2$ (pH adjusted with NaOH) and allowed to fight as described above ($N=3$ trials for each pairing). All 2 mmol $l^{-1}$ $CoCl_2$-treated fish ate almost immediately after treatment, showed normal swimming behavior, had no excess mucus production and had normal coloration. It is also important to note that no fish died as a result of the 3 h, 2 mmol $l^{-1}$ $CoCl_2$ treatment ($N=40$), and that a 4 mmol $l^{-1}$ $CoCl_2$ treatment for 3 h is the lethal dose for *A. burtoni*.

During verification of our lateral line system ablation treatments, we noted reduced DASPEI staining in the olfactory epithelium compared with control and sham fish, suggesting that 2 mmol $l^{-1}$ $CoCl_2$ treatment may also impair chemosensory systems. To ensure that any behavior differences were not due to impaired olfaction, we used a micro-cauterizer (Cautery High Temp Adjust-A-Temp Fine Tip, Bovie Medical Corporation, Clearwater, FL,

USA) to ablate the olfactory epithelium (anosmic fish) prior to placing fish in the same experimental paradigm used for our mechanosensory trials ($N=3$). Fish were allowed to recover for 3 days prior to the forced territorial interaction, and behavior from $CoCl_2$-treated, EGTA-treated and anosmic control fish were then compared with behavior of fish from both control and sham trials.

### Statistical analyses

Data were analyzed in SigmaPlot 12.3 (Systat Inc., San Jose, CA, USA). Comparisons between fight winners and losers were made with a Student's *t*-test or Mann–Whitney *U*-test if normality assumptions were not met. Square root and log transformations were used to normalize data if needed. Comparisons among trial groups were made with one-way ANOVA followed by *post hoc* Student–Newman–Keuls (SNK) tests for pairwise comparisons (when normality passed), or Kruskal–Wallis (KW) ANOVA on ranks followed by *post hoc* Dunn's test for multiple comparisons (when normality failed and data could not be normalized by transformation).

Student's *t*-tests and Pearson correlations were used to compare winner and loser behaviors. Because there was no difference between winners and losers ($P>0.05$ for all comparisons) and winner and loser behaviors were strongly correlated ($P<0.05$ for all behaviors), a composite score (winner and loser added together or averaged) for each category was used to avoid pseudo-inflating sample size or influencing statistical tests.

## RESULTS

### Characterization of the *A. burtoni* lateral line system

A generalized distribution map of the canals and neuromasts of the *A. burtoni* lateral line system was created using cleared and stained fish, with Methylene Blue injections to visualize canals and pores, and DASPEI staining to identify neuromasts (Fig. 2A). The *A. burtoni* lateral line system is similar to the typical teleost lateral line system with seven cranial canals: infraorbital (IO), supraorbital (SO), preopercular (PR), mandibular (MD), postotic (PO), otic (OT)
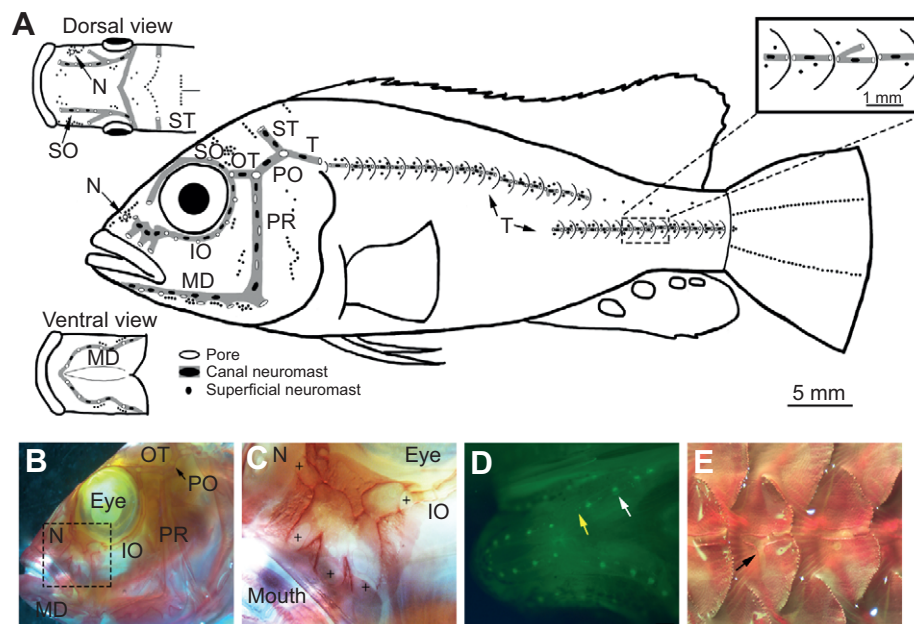


**Fig. 2. Distribution and morphology of the mechanosensory lateral line system in *A. burtoni*.** (A) The *A. burtoni* lateral line system is similar to that of other cichlids with seven main cranial canals and a disjunct trunk canal. Canal neuromasts (black ovals) are located inside canals (gray shading) under the skin between pores (open ovals). Superficial neuromasts (black dots) are located around each naris, in rows or clusters in the vicinity of canals, and in two rows along the length of each side of the caudal fin. (B) Canal structures can be seen clearly in cleared and stained fish, including the wide tubules of the infraorbital canal. (C) Higher magnification image of the boxed portion of B showing tubules that extend toward the mouth. The plus sign indicates canal pores just above the mouth and beneath the eye. (D) Ventral view of DASPEI-stained fish to illustrate labeling of both superficial (yellow arrow) and canal neuromasts (white arrow) in the mandibular canal region. (E) Although the trunk canal is typically unbranched, occasional side tubules (arrow) from the trunk canal were found on some scales in some fish. IO, infraorbital; MD, mandibular; N, naris; OT, otic; PR, preopercular; PO, postotic; SO, supraorbital; ST, supratemporal; T, trunk. Scale bars: B, 1 cm; C, 0.5 cm; D, 1 cm; and E, 200 μm.

and supratemporal (ST) canal. The SO canal runs down the dorsal part of the head and ends in a pore just prior to the upper lip, and has a tubule that branches laterally to surround the eye and terminates in a pore just above each naris. Typically, a CN was located between adjacent pores and each canal had nearby clusters or lines of SNs (Fig. 2D). The MD canal on the ventral side of the lower jaw is continuous across the midline to connect the left and right sides, while tubules of the SO canal on the dorsal portion of the head also connect the two sides of the fish. The OT and PO canals serve as links between IO and SO, PR and trunk (T) canals. Like most cichlids, *A. burtoni* possesses a disjunct trunk lateral line canal (Webb, 1989), with a dorsally located rostral portion and a medially located caudal portion. Each trunk scale has a single CN with several (1–6, but most commonly 2–3) SNs. Although some tubules branched off the main trunk canal (tubules found on 2–3 scales per fish; see Fig. 2A inset, Fig. 2E), a single straight canal was most common. The caudal fin had 2–3 small SNs in a cluster at the base of the caudal fin and two straight lines of SNs extending down both sides of the caudal fin. A ring of SNs also surrounded each naris.

The distribution and number of neuromasts was similar between males and females (Table 2; Student's *t*-test, $P>0.05$ for all comparisons). The only exception was that males had more SNs located on their caudal fins than did females (fish with part of the caudal fin missing excluded; *t*-test, $t=-3.790$, d.f.=7, $P=0.016$). Fry (12 dpf; total length 12.58±0.27 mm) had neuromasts located in typical adult canal locations, but the neuromasts were on the skin and not yet enclosed in canals. The only adult-typical SNs present in the 12 dpf fry were those surrounding the nares.

### Role of the mechanosensory lateral line system in territorial interactions

#### Pre-fight and non-fight behaviors

To test the role of the lateral line system in territorial interactions, we used a behavioral paradigm in which two dominant males had equal opportunity to acquire a new territory (Fig. 1). For a fight to occur, one fish must initiate a fight and the opponent has the option to fight back if he determines that the benefits of winning the territory (e.g. spawning substrate) outweigh the possible costs of losing a fight (e.g. physical damage, reproductive suppression). These conditions resulted in three possible outcomes: no fight occurred within the 30 min trial, one fish became aggressive while the other became subordinate (no-contest win), or a fight occurred. Eleven of the 15 (73.33%) trials between two sham fish and 9 of 14 (64.29%) trials

between one sham and one ablated fish, but only 9 of 20 (45%) trials between two ablated fish resulted in a fight within the trial time (Fig. 3A). A no-contest win occurred in 2 of 15 (13.33%) trials between two sham fish, 4 of 13 (30.76%) trials between a sham and an ablated fish but only 1 of 20 (5%) trials between two lateral line-ablated fish. Interestingly, the sham fish won all of the no-contest wins in trials with one sham and one ablated fish. The last category (no fight during trial time) occurred predominantly during trials between two lateral line-ablated fish: 2 of 15 (13.33%) trials between two sham fish, only 1 trial between a sham and an ablated fish (7.69%), but 10 of the 20 (50%) trials between two ablated fish ended with no fight occurring. By assigning a latency time of 30 min for these no-fight trials, trials between two ablated fish had a longer latency to fight onset when compared with trials involving a sham fish (KW, $H=7.693$, d.f.=2, $P=0.021$; Dunn's, $P<0.05$; Fig. 3B).

Seven of the 8 (87.5%) fights between a sham and ablated fish were initiated by the sham fish (Fig. 3C). The probability of this occurring is 3.125%, suggesting that this was not random chance. Using time spent within 1 BL (the effective range of the lateral line system; Braun and Coombs, 2000) of the opponent as a measure of time assessing the opponent, fish in trials with a lateral line-ablated fish spent significantly more time assessing their opponent than did sham fish in trials between two sham fish (KW, $H=13.411$, d.f.=2, $P<0.001$; Dunn's, $P<0.05$; Fig. 3D). While this measure does not distinguish between the sham and ablated fish in the sham–ablated pairing, it should be noted that the ablated fish was often observed following the sham fish around the tank while the sham fish occasionally performed aggressive behaviors directed at the ablated fish.

#### Fight behaviors

A ratio of contact to non-contact behaviors was generated by dividing the total number of contact behaviors by the total number of non-contact behaviors. A number >1 signifies a tendency for contact behaviors while <1 signifies use of predominantly non-contact behaviors. Lateral line-ablated fish in trials between two ablated fish had a significantly higher contact to non-contact ratio than fish from trials between two sham fish or a sham and ablated fish (ANOVA, $F_{2,51}=32.547$, $P<0.001$; SNK multiple comparison: $P<0.001$; Fig. 4A). Fish in trials between two sham fish and between one sham and one ablated fish used predominantly non-contact behaviors (0.331±0.049; mean±s.e.m.). In contrast, lateral line-ablated fish in trials between two ablated fish used primarily contact behaviors (1.893±0.403). Fights between two sham fish began with mostly

**Table 2. Quantification of neuromasts in female, male and 12 dpf *A. burtoni***

| | Females (*N*=6) | Males (*N*=6) | 12 dpf fry (*N*=4) |
|---|---|---|---|
| Rostral trunk CNs | 13–19 (17.00±1.01) | 16–18 (17.00±0.35) | 17–19 (18.0±0.41) |
| Caudal trunk CNs | 9–13 (10.38±0.66) | 10–13 (11.6±0.57) | 10–12 (12.5±0.29) |
| Mandibular canal CNs | 4 | 4 | 4 |
| Preopercular canal CNs | 7 | 7 | 7 |
| Infraorbital canal CNs | 6 | 6 | 6 |
| Mouth canal CNs | 3 | 3 | 3 |
| Supraorbital canal CNs | 4 | 4 | 4 |
| Rostral trunk SNs per CN | 2–3 (2.31±0.16) | 1–6 (2.06±0.25) | Not developed |
| Caudal trunk SNs per CN | 1–3 (1.75±0.25) | 1–3 (1.81±0.06) | Not developed |
| No. of rows on caudal fin | 2 | 2 | Not developed |
| Caudal fin SNs per row | 10–25 (19.5±2.70) | 25–47 (40.2±4.44)* | Not developed |
| SNs surrounding naris | 6–15 (9.83±1.73) | 9–15 (11.20±1.24) | 5–6 (4.5±0.29) |

CN, canal neuromast; SN, superficial neuromast; dpf, days post-fertilization.
Values are presented as a range of the number of quantified neuromasts, and means±s.e.m. are given in parentheses. Single values represent neuromast number with no variation among individuals. The disjunct trunk canal was split into the rostral and caudal portions for quantification purposes. The 'mouth canal' consists of the tubules extending from the infraorbital canal towards the mouth. No differences were observed between female and male fish except for the number of superficial neuromasts per row on the caudal fin (*), which was greater in males.
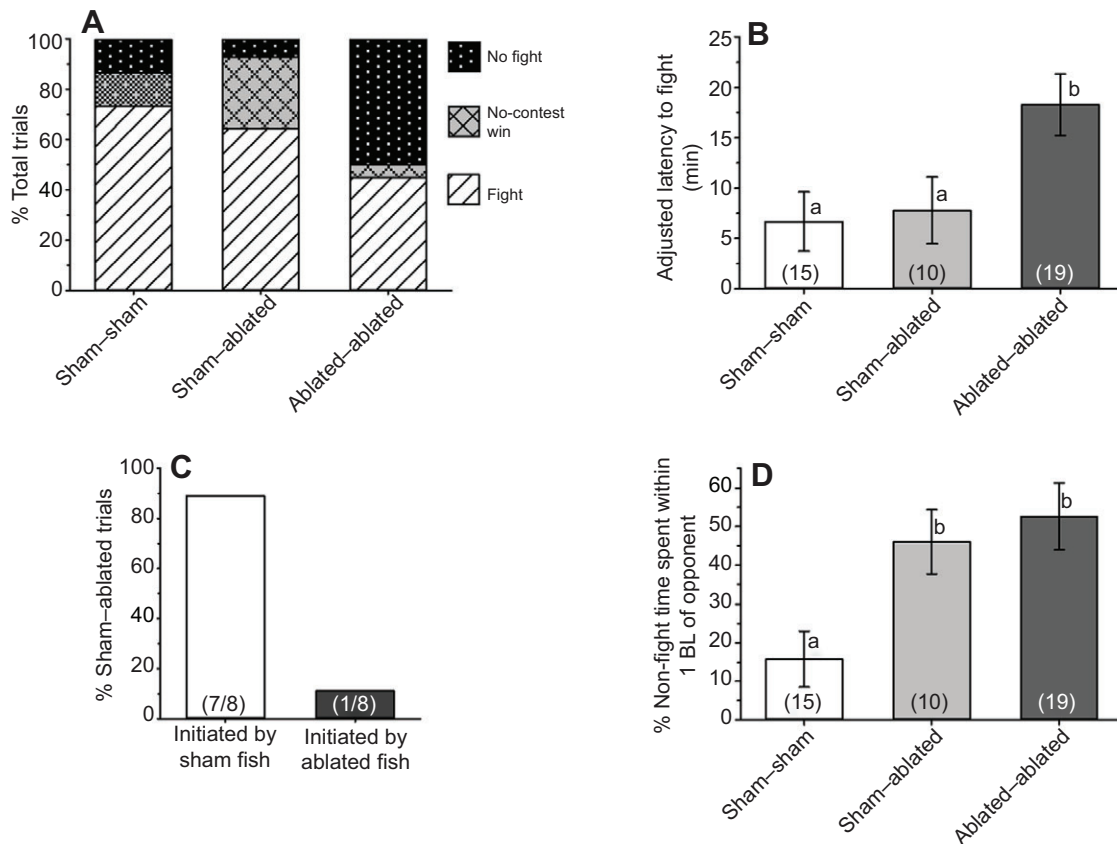
**Fig. 3. Lack of mechanosensory input impairs fight motivation and opponent assessment in male *A. burtoni*.** (A) When lateral line-ablated fish interacted with other ablated fish, fewer trials resulted in a fight than when sham-handled fish interacted with other sham fish or with ablated fish (45%, 64.29% and 73.33%, respectively). (B) By assigning a latency time of 30 min to trials in which no fight occurred, the latency to initiate a territorial interaction was longer in trials that had two lateral line-ablated fish (ablated–ablated) (ANOVA: $P$=0.021; no-contest wins excluded from analysis). (C) During trials where sham fish were paired with lateral line-ablated fish, the sham fish initiated the fight 7 out of the 8 times (87.5%), which differs from random chance. (D) In trials that had at least one ablated individual (sham–ablated and ablated–ablated pairings), fish spent significantly more of the non-fight time within 1 body length (BL) of their opponent compared with trials where both fish had intact lateral lines (sham–sham) (Kruskal–Wallis, $P$<0.001). Data in B and D are plotted as means±s.e.m. and sample sizes are given in parentheses. Different letters indicate significant differences among groups at $P$≤0.05.

non-contact behaviors and escalated to the use of some contact behaviors over time (Fig. 4B). In trials between two ablated fish, however, lateral line-ablated fish began with contact behaviors and shifted to non-contact throughout the trials (Fig. 4C). Further, only 1 of all 30 sham-handled fish performed an abnormal lateral display (physically touching the opponent as opposed to being within 1 BL but not physically touching), whereas 17 of all 26 lateral line-ablated fish performed abnormal lateral displays (Fig. 4D).

To test whether aggression levels varied depending on lateral line function, we calculated an aggressive score for each fish (total number of aggressive behaviors divided by the fight duration), and winner and loser scores were combined to have an aggressive score per trial. Aggression levels did not differ based on the presence or absence of lateral line function (KW, $H$=4.059, d.f.=2, $P$=0.131; Fig. 5A). Fight duration did not differ between trial pairings (ANOVA, $F_{2,25}$=1.171, $P$=0.372; Fig. 5B), and lateral line ablation did not affect the ability of a fish to win a fight (Fig. 5C). Of the 8 fights between one sham and one ablated fish, sham fish won 3 and ablated fish won 5 trials, suggesting that lack of hydrodynamic cues does not affect a fish's ability to win a territorial fight.

**Sham-handling and control treatments do not affect behavior**
To ensure that any observed behavioral differences were due to the lack of hydrodynamic cues associated with ablating the lateral line

system and not to handling stress or treatment toxicity, we included a variety of control trials (e.g. cobalt control, EGTA control, anosmic control). No difference in latency to fight or fight duration was observed between any of the control pairings (ANOVA; latency: $F_{4,27}$=0.308, $P$=0.870; fight duration: $F_{4,27}$=0.876, $P$=0.491). There was also no difference between the number of aggressive behaviors per minute among control treatments (KW: $H$=5.471, d.f.=4, $P$=0.242) indicating that treatment (i.e. cobalt, EGTA) and elimination of olfactory cues do not impact overall aggression. In addition, control treatments had no impact on the ratio of contact to non-contact behaviors (ANOVA, $F_{4,59}$=0.758, $P$=0.557) or the time spent within 1 BL of the opponent (KW, $H$=0, d.f.=4, $P$=1.00), indicating that treatment handling and toxicity did not influence the behavioral preference of fish or their ability to assess opponents. Based on these data, we only compared behavior data from lateral line-ablated fish with those from sham-handled fish in the previous sections.

**DISCUSSION**
We sought to determine whether use of the mechanosensory lateral line system was important for territorial interactions in the African cichlid fish, *A. burtoni*, and whether the morphology of the lateral line system differed between sexes. We mapped the canal and neuromast distribution of the *A. burtoni* lateral line system and found that the
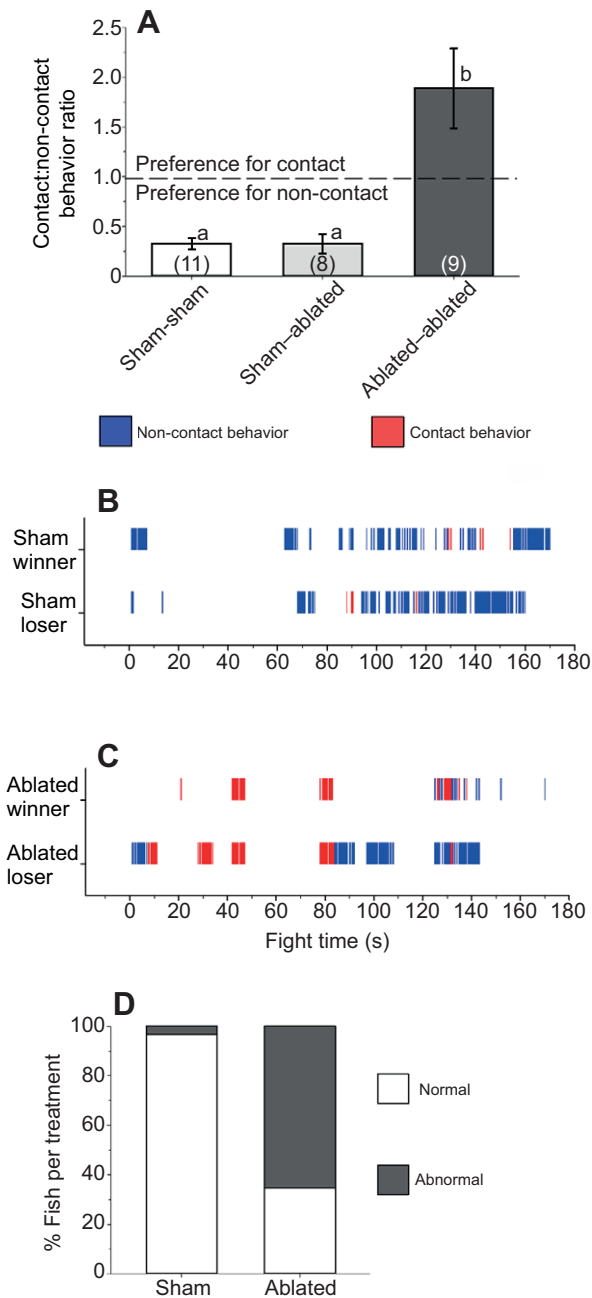
**Fig. 4. Lateral line-ablated fish have altered fight behaviors.** (A) Fish in ablated–ablated trials had a higher contact to non-contact behavior ratio (total number of contact behaviors/total number of non-contact behaviors) than did fish in sham–sham and sham–ablated trials (ANOVA, $P<0.001$). Data are plotted as means±s.e.m., sample size is given in parentheses, and different letters indicate significant differences among groups at $P\leq0.05$. (B,C) Raster plots of contact and non-contact behaviors during the trial fight time show a difference in the sequence of behavioral patterns. Fish in sham–sham trials (B) use primarily non-contact behaviors and escalate to some contact behaviors over time. In contrast, fish in ablated–ablated trials (C) use mostly contact behaviors from the start of the trial. (D) Only 3.34% of sham-handled fish performed abnormal lateral displays (lateral display that occurs with physical contact to the opponent), while 65.38% of ablated fish performed abnormal lateral displays.

number of neuromasts was consistent across sexes, social status and reproductive state. By chemically and physically ablating the lateral line system in dominant males prior to forced territorial interactions, we also show that hydrodynamic cues are used to assess opponents
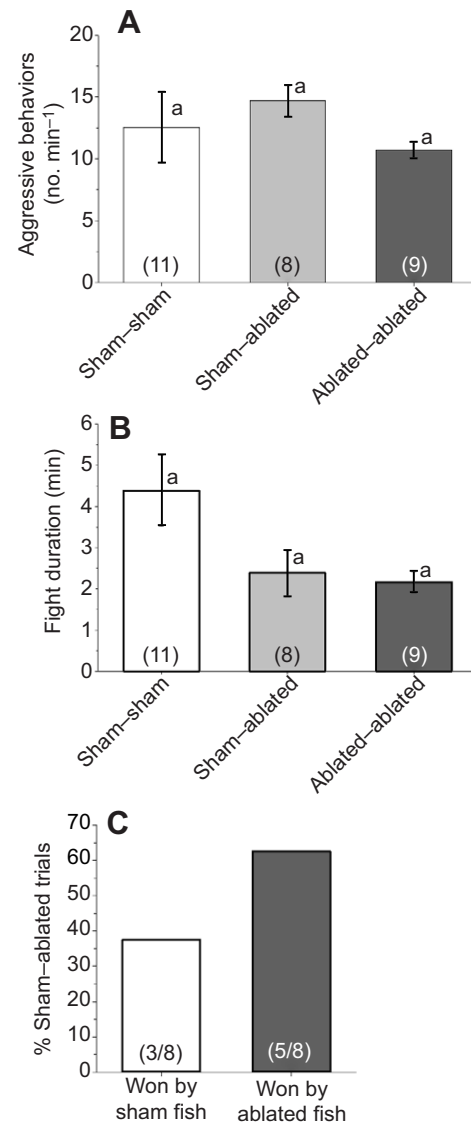


**Fig. 5. Ablation of the lateral line system does not alter overall aggressiveness, fight duration or the ability to win a fight.** (A) Lateral line ablation did not alter the total number of aggressive behaviors performed per minute (Kruskal–Wallis, $P=0.131$). (B) Fight duration was not affected by loss of lateral line function (ANOVA, $P=0.372$). (C) Lack of lateral line function did not impair a fish's ability to win a fight. Sham fish won 3 of 8 trials while ablated fish won the other 5. Data in A and B are plotted as means±s.e.m. and sample sizes are given in parentheses. Same letters indicate no significant differences among groups at $P>0.05$.

and to facilitate the use of less dangerous non-contact aggressive behaviors. Our data suggest that the use of the lateral line system for reception of hydrodynamic cues associated with territorial behaviors is a protective mechanism to allow fish the opportunity to assess the opponent without engaging in direct, and potentially damaging, physical contact. This is the first direct experimental evidence in any species that mechanosensory information detected by the lateral line system plays an important role in aggressive social interactions.

### Morphology of the mechanosensory lateral line system
Lateral line canal morphology in *A. burtoni* is consistent with that described for several other cichlid species (Greenwood, 1981). Canal morphology is similar to that of the Lake Malawi cichlids *Tramitichromis* sp., *Labeotropheus fuelleborni* and *Metriaclima*

*zebra*, all of which are classified as having narrow canals (Bird and Webb, 2014; Webb et al., 2014). While *A. burtoni* are found in shallow shore pools of Lake Tanganyika, genetic techniques have classified them as a riverine species (Brawand et al., 2014), and the narrow canal morphology is consistent with river-dwelling fish. The more turbulent environment of rivers creates hydrodynamic noise, and the narrow canal morphology and small pore diameter help reduce noise to better detect relevant signals (Klein and Bleckmann, 2015).

The distribution of cranial neuromasts in *A. burtoni* is consistent with all previously examined African cichlids, independent of whether they possess widened or narrow canals (Webb et al., 2014; Bird and Webb, 2014). We did not observe any major neuromast differences between males and females, or between social and reproductive states. Although the overall number of neuromasts did not differ between reproductive states, it is possible that neuromasts could have increased sensitivity during reproductively active states, particularly in females, as hydrodynamic cues are produced during body quivers and tail waggle behaviors performed by courting males. For example, aromatase and estrogen receptors are necessary for neuromast development and continue to be expressed into adulthood (Froehlicher et al., 2009; Hamilton et al., 2014; Tingaud-Sequeira et al., 2004). It is possible, therefore, that sex steroid receptors in neuromasts modulate mechanosensory function similar to that described for the inner ear of fish (Maruska and Fernald, 2010a; Maruska and Sisneros, 2015; Sisneros et al., 2004), but this requires further study.

In our population of *A. burtoni*, fry are typically released from the female buccal cavity at ~12 dpf when the lateral line system canals are still developing. Although we found most neuromasts were present, canals were not completely formed at this point. This is consistent with ontogenetic studies of other cichlids in which canal ossification does not occur until 42–56 dpf (Webb et al., 2014), but future studies are needed to fully examine the morphological development of the *A. burtoni* lateral line system.

### The lateral line system is used to assess opponents
Lateral line-ablated fish spent more time within 1 BL of their opponents, suggesting that the lateral line system facilitates assessment of opponents during male–male territorial interactions in *A. burtoni*. Lateral line-ablated fish were less inclined to start territorial fights and more likely to forfeit a fight. This decreased fight motivation was likely due to an impaired ability to adequately assess the opponent. Models of animal assessment range from pure self-assessment to pure mutual assessment but it is likely that animals use a combination of self and mutual assessment throughout an agonistic interaction (Arnott and Elwood, 2009; Enquist et al., 1990; Elias et al., 2008; Hofmann and Schildberger, 2001; Hsu et al., 2008; Neat et al., 1998). For instance, Hsu et al. (2008) suggested that animals rely on mutual assessment to determine when to escalate a fight, but then switch to self-assessment to determine how long to sustain the fight. The mutual assessment phase is when animals use non-dangerous behaviors and close-range sensory modalities to gauge the potential threat of an opponent. These behaviors can range from circling and sniffing in rodents (Gosling and McKay, 1990) to antenna fencing in insects (Hofmann and Schildberger, 2001). We propose that in *A. burtoni*, mutual assessment is a multimodal behavioral task dependent on visual and hydrodynamic cues (and potentially chemical and acoustic cues, although not examined here). In typical cichlid fights, the fight is initiated by a non-contact behavior, normally a lateral display. During lateral displays, one fish flares its fins and gently

quivers its body while the opponent is oriented perpendicular to him. This orientation and behavior maximizes the production and reception of both visual and hydrodynamic cues. Fish will alternate roles between sender and receiver, until the fight escalates to include contact behaviors (e.g. bites, mouth fights). Fights between two lateral line-ablated fish did not have the initial lateral display circling behaviors observed in typical cichlid fights (based on our experiments and those of Enquist et al., 1990). This, combined with the decreased fight motivation in lateral line-ablated fish, suggests that hydrodynamic cues are important for fish assessment. When input to the lateral line system is eliminated, fish cannot properly assess their opponent and instead are more likely to forfeit a fight. Crickets with removed antennal input have highly reduced male–male aggression, suggesting that both chemosensory and mechanosensory input are critical to their mutual assessment (Murakami and Itoh, 2003). Similarly, hydrodynamic cues appear to be essential for mutual assessment in *A. burtoni*, and potentially in other territorial fishes.

Decreased fight motivation in *A. burtoni* is only seen when engaging in a fight to acquire a new territory as opposed to defending an already acquired territory. When used in a resident–intruder paradigm, all lateral line-ablated resident fish immediately defended their territory when exposed to lateral line-intact or ablated intruders (J.M.B., unpublished observations), suggesting that the decreased fight motivation observed in our experiments is unique to acquiring a new territory and likely due to impaired assessment abilities and not decreased aggression. This suggests that although similar behaviors are used in each interaction, fish view these as different behavioral scenarios. In crayfish, these two behavioral scenarios are often linked, with the most dominant crayfish being the quickest to acquire a territory and perform the most evictions (territory defense behavior) (Fero and Moore, 2008; Martin and Moore, 2008). However, it is possible that these two social situations may be perceived and processed differently in the brain, similar to how affective and predatory aggression have different neural pathways in mammals (Gregg and Siegel, 2001). Although the causes of differences in threat perception between these two scenarios are unknown, the territorial defense behaviors towards an intruder used by lateral line-ablated fish indicate that lack of hydrodynamic cues only impacts assessment ability and not overall aggression.

Many fish species use visual cues during social encounters to gain insight into their perceived social standing (Chen and Fernald, 2011; Grosenick et al., 2007; Korzan and Fernald, 2007), but few studies have isolated visual cues without other sensory systems to test the importance of visual cues alone. However, Chen and Fernald (2011) found that visual cues alone were sufficient for male fish to assess others and make the appropriate behavioral decision, but it is important to note that these effects were seen only when one fish was ~4 times larger than the other. In our experiments, fish were size matched (<10% size difference) and able to use visual cues, yet we still saw noticeable differences in assessment capabilities (e.g. time within 1 BL) when the lateral line system was ablated. This suggests that visual cues alone are not enough to properly assess an opponent when closely size matched, and that fish assessment, like many other cichlid fish behaviors, is dependent on multimodal sensory inputs and not just vision.

### Lateral line-ablated fish have altered fight behaviors
*Astatotilapia burtoni* males use a variety of behaviors, such as lateral displays, frontal threats, mouth fighting and biting, when engaged in a territorial fight. These behaviors always occur within

1–2 BL of the opponent and create water movements that should activate the opponent's lateral line. Although previous studies suggested possible involvement of mechanoreception during agonistic interactions in fishes (Enquist et al., 1990; McMillan and Smith, 1974; Montgomery et al., 2014), our study is the first to directly show that the lateral line system is used in fight behaviors. We classified behaviors as either contact (physical contact occurred) or non-contact (within 1 BL but without physical contact) and showed that lateral line-ablated fish used predominately contact instead of non-contact behaviors. It is possible, therefore, that lateral line system inputs help mediate decisions to perform appropriate agonistic behaviors, such that without the reception of hydrodynamic cues, fish choose to use contact instead of non-contact behaviors. The use of non-contact instead of contact behaviors is important for a couple of reasons. First, these non-contact behaviors act as aggressive displays with less risk of physical damage, and second, they allow fish to be more aware of their surroundings. For example, the South American cichlid *Nannacara anomala* was slower to detect approaching predators when engaged in contact behaviors compared with non-contact behaviors (Jakobsson et al., 1995), suggesting that non-contact behaviors may act as a self-preservation technique. Without lateral line system input, fish cannot use non-contact behaviors for mutual assessment, causing them to proceed directly to more costly contact behaviors that can be perceived by the somatosensory system.

In *A. burtoni*, we also saw more abnormal lateral displays (performed with physical contact to the opponent) in lateral line-ablated fish. This could be caused by the ablated fish's inability to perceive water movements and identify an opponent's location, thereby accidently causing contact. It is also possible that the opponent initiates the contact, because without detection of water movements from an approaching fish, the ablated fish may not be able to quickly react and evade the bite. While we cannot distinguish between these different possibilities, future research should examine areas of the brain implicated in social decision making to determine the relative importance of hydrodynamic inputs from the mechanosensory lateral line system in mediating adaptive behavioral decisions during territorial contests.

Although lateral line system ablation in *A. burtoni* affected the type of behaviors performed (e.g. contact versus non-contact) during territorial interactions, overall aggression, fight duration and fight outcome were not impacted. These data suggest that ablated fish simply replaced the use of non-contact behaviors with contact behaviors, without significant consequences on fight outcome. This supports the idea that once a fight escalates to contact behaviors, fish rely more on self-assessment (Hsu et al., 2008), while fight duration and outcome are more dependent on a fish's perceived standing in the fight and damage incurred throughout the fight. Thus, hydrodynamic cues detected by the lateral line system likely facilitate mutual assessment and the use of non-contact fight behaviors, but have no impact on self-assessment or the ability to win a fight.

### Cobalt chloride treatment

To effectively ablate the entire mechanosensory lateral line system in *A. burtoni*, we used a combination of 2 mmol l$^{-1}$ CoCl$_2$ treatment and transection of the posterior lateral line nerve (see Materials and methods). Although others have used this cobalt concentration (Medina et al., 2013; Montgomery et al., 1997), it has been scrutinized (Janssen, 2000) for its high dosage and potentially toxic effects. To address this, we included several controls to verify that

the treatment was not toxic to *A. burtoni*. These controls included treatment with 1 mmol l$^{-1}$ EGTA (used to lower calcium levels in the water) and 2 mmol l$^{-1}$ CoCl$_2$ in calcium-rich water. Neither of these treatments impaired the lateral line system or altered behavior when compared with control and sham-handled fish. Because cobalt is a non-specific calcium channel antagonist, we also checked for potential effects on other exposed sensory systems. DASPEI staining of the olfactory epithelium was noticeably reduced after treatment (observed via decreased fluorescence, see Fig. 1), suggesting that CoCl$_2$ likely also impairs olfaction, and future studies will evaluate the extent of cobalt effects on olfactory function. However, because of this observation, and the fact that *A. burtoni* uses chemosensory signaling via urine release in male–male interactions (Maruska and Fernald, 2012), we also examined behavior of anosmic (olfactory epithelium ablated) fish to verify that any behavioral differences we observed in lateral line-ablated fish were not due to impaired olfaction. These trials showed that anosmic fish did not differ behaviorally from control and sham-handled fish, indicating that lack of mechanosensory rather than olfactory input in lateral line-ablated fish was responsible for the observed behavioral differences. We did not ablate the taste or solitary chemosensory cells because it is not feasible, but neither has been implicated in social communication. Thus, we are confident that the observed behavioral differences were due to lack of hydrodynamic inputs and not chemosensory inputs. Fish responded to visual stimuli, indicating that the cobalt treatment did not affect vision, and we have no reason to suspect that cobalt treatment affected hair cells of the inner ear.

Despite the unwanted, but controlled for, effects on the olfactory system, we are also confident that the treatment had no toxicity effects. Fish could be housed in the cobalt solution for several days without any ill-effects, no fish died as a result of the treatment, and all animals used in behavioral trials were alive and behaving normally for months after the treatment. In addition, all treated fish had normal swimming and feeding behaviors. There are many possible reasons why our fish required a higher dose of cobalt than many other species. Aminoglycosides are already known to be highly species specific (Brown et al., 2011), with some species not responding to treatment at all. Cobalt treatment is also likely species specific, and the use of salts in our water to mimic the low salinity environment of Lake Tanganyika potentially added ionic interference. Based on our experience with *A. burtoni* and the species-specific variations of lateral line system ablation treatment methods in other species (Brown et al., 2011), we propose that researchers using a chemical or pharmacological ablation approach should verify that their treatment is effective for their particular species using a vital dye such as DASPEI, rather than relying on published treatment regimes for other species. Those using cobalt should also test, and then control for, any comorbid effects on chemosensory systems, especially when examining the role of the lateral line system in a multimodal behavior, such as prey detection, predator avoidance or social communication. These controls are crucial for testing the relative role of the lateral line system in any behavioral context.

### Summary

Use of the mechanosensory lateral line system during territorial behaviors in fishes has long been suggested (Enquist et al., 1990), but never directly tested. To our knowledge, ours is the first evidence in any fish species that hydrodynamic cues detected by the lateral line system are used to assess opponents during agonistic interactions. In addition, animals lacking lateral line input relied

predominantly on contact over non-contact behaviors, suggesting that mechanoreception facilitates the use of non-contact behaviors as a protective mechanism during territorial interactions. Contact behaviors are inherently more dangerous, and the use of non-contact behaviors allows fish to send and receive information, and potentially resolve conflicts, without physical damage. While the lateral line system has largely been studied in terms of its role in feeding behavior, our study demonstrates that mechanosensory information is also used in territorial interactions and should be considered when studying social behaviors involving multimodal sensory input.

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