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Urination and Aggravation: Status-Relevant Visual and Chemosensory Signals Escalate Aggressive Interactions in an African Cichlid Fish

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by

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ABSTRACT

Sensory processing of communication stimuli is essential for the survival of organisms across all evolutionary branches. Multimodal signaling, the use of multiple sensory systems, is crucial in this process but little is known about the relative importance of different senses used during aggression and where this salient information is integrated within the brain to make adaptive behavioral decisions that dictate survival and species persistence. I used the African cichlid fish, *Astatotilapia burtoni*, to test how visual and chemosensory signals in male-male interactions influences male behavior and neural activation patterns. Males of this species exist in a dominance hierarchy, where brightly-colored dominant individuals aggressively defend territories they use for reproductive activities. I presented focal males with visual and chemosensory signals from other males either alone (unimodal) or together (multimodal) and found that vision is necessary for males to engage in aggressive behaviors such as frontal displays, lateral displays, and border fights. However, aggressive behaviors were dramatically increased when focal males received both visual and chemosensory signals from rival males, suggesting that male-released chemical signals are also important for territorial interactions. Using the immediate early gene *cfos* as a proxy for neural activation, I identified several brain regions involved in processing unimodal and multimodal visual-chemosensory information in male-male territorial contexts. This study is the first to examine how visual-chemosensory signaling impacts male-male aggressive behavior in *A. burtoni* and provides insight on how these signaling modalities mediate territorial interactions and where in the brain this information is processed.

INTRODUCTION

Sensory processing of environmental stimuli is necessary for the survival of organisms across all evolutionary branches. Communication signals sent via different sensory modalities, such as vision, chemosensory, touch, smell, and sound typically convey distinct types of information, are often delivered together, and consequently elicit context-dependent behaviors in a receiving animal (Bradbury & Vehrencamp, 2000). These stimuli are detected by multiple sensory systems (multimodal), but little is known about where salient sensory information is integrated within the brain to make adaptive behavioral decisions that dictate survival and species persistence (S. Partan & Marler, 1999).

Visual-chemosensory multimodal communication is widespread throughout the animal kingdom, with examples in both vertebrates and invertebrates (Kotrschal, 2000; Griffith & Ejima, 2009; Isogai et al., 2011). While vision is the dominant sense that mediates aggressive behaviors in most species, chemosensory communication is also commonly used across the animal kingdom to signal social rank. For example, male tilapia fish, *Oreochromis mossambicus,* actively signal their dominance status via odorants released in the urine which may modulate aggression levels in conspecific males (Barata et al., 2007). However, the neural links between multisensory inputs and receiver behavioral output remains poorly understood (S. R. Partan, 2013; Ronald et al., 2012). Further, the physiology and/or reproductive state of receivers can influence how such sensory signals are processed (Insel, 2010; Maruska & Butler, 2021). Thus, examining communication from a perspective that goes beyond behavioral responses to include receiver physiology

and neural processing mechanisms is crucial for understanding the function and evolution of context-dependent signaling.

Astatotilapia burtoni, a species of African cichlid fish, is an important model for studying communication through both multimodal and single unimodal sensory perception (Maruska and Butler, 2021). Males of this species (**Figure 1**) are socially ranked as either dominant or subordinate; dominant males are brightly colored, establish and defend territories, and spawn with females, whereas subordinate males are dull in coloration, lack territories, and do not spawn with females (Maruska & Fernald, 2014). Males defend territories from rival males through aggressive behaviors typified by chasing, biting, gill flaring, shaking their body, and erecting their fins to appear larger. In addition to these visual displays, males increase their urination in the presence of another male (Maruska & Fernald, 2012), but the impacts of this chemosensory signal on aggressive behavior is not known. Visual-chemosensory signaling is important in male-

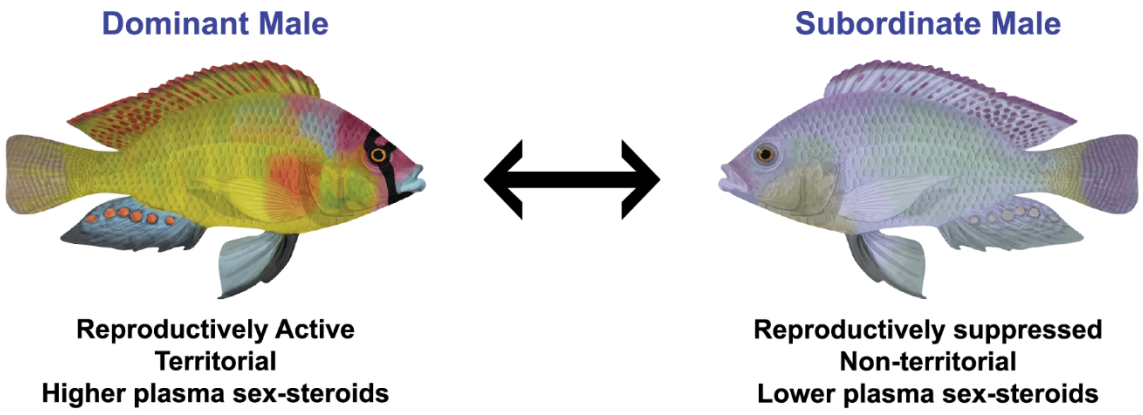

Figure 1 | The males of the African cichlid fish, *Astatotilapia burtoni*, exist within a social hierarchy. Dominant males (left) are brightly colored with distinct yellow-orange egg spots on their anal fins. In addition, there are dark forehead stripes, a dark opercular spot on the caudal edge of the gill cover, a stripe (eye bar) extending through the eye to the lower jaw, and a bright orange-red patch on the humeral scales. Subordinate males (right) are dull in coloration and are more similar to females in coloration.

female *A. burtoni* reproductive interactions (Field et al., 2018), but how these signaling modalities affect male-male aggression is unknown. Because territory ownership directly dictates reproductive fitness, understanding which sensory signals mediate male-male interactions in this species is important.

How relevant sensory and social information is integrated with an organism's internal physiology to elicit context-dependent behaviors is one of the main goals of behavioral neuroscience (Insel, 2010). The social decision-making network (SDMN) is a collection of highly conserved brain nuclei proposed as a framework for examining where and how this salient information leads to adaptive behaviors (Newman, 1999; O'Connell & Hofmann, 2011), but it is increasingly clear that many brain regions outside of this network are also involved in the social-decision making process. By associating specific behavioral outputs with neural activation patterns of receivers in response to unimodal and multimodal signals, we can contribute to the limited knowledge of how multimodal sensory signals are processed in the brain of receivers to induce behavioral responses. Further, little is known about where socially-relevant chemosensory signals are processed in the brain, especially in fishes (Nikonov & Caprio, 2005; Yabuki et al., 2016; Yaksi et al., 2009). By examining neural activation patterns in socially-relevant brain regions as a complement to behavioral responses in receivers, we can expand upon the provided framework to advance the current knowledge of neural substrates that link sensory inputs to behavioral outputs.

The overall goal of my research was to understand how visual and chemosensory signaling influences behaviors and neural activation patterns during male-male cichlid

contests. To investigate how dominant males respond to unimodal and multimodal visual-chemosensory aggressive signals, I exposed dominant males to visual and chemosensory signals from other dominant males either alone or combined, and recorded males' behavioral and physiological responses. Further, I used *in situ* hybridization for the immediate early gene *cfos* to test the hypothesis that neural activation patterns differ among males receiving unimodal and multimodal signals from opposing dominant males. My results demonstrate that visual signaling is required in *A. burtoni* male-male contests, but presence of chemosensory information increases aggressive behaviors. Preliminary results of neural activation patterns also suggests that vision is dominant, and that unimodal chemosensory and multimodal visual-chemosensory information is processed differently within several SDMN regions known to be involved in aggressive behaviors. This research will advance our understanding of multisensory processing mechanisms in the vertebrate brain by revealing how visual and chemosensory information are used by dominant males during territorial contests and by identifying specific brain regions important in processing these senses.

MATERIALS AND METHODS

Experimental Animals

Adult *Astatotilapia burtoni*, originally collected from Lake Tanganyika, Africa, were bred in laboratories since the 1970s and display similar behaviors to those in wild populations. The lab bred stock is kept in aquaria under temperature, water, and lighting conditions similar to their natural habitat (28 ºC; pH 8.0; 12 h light: 12 h dark cycle). Aquaria contained halved terra cotta pots to serve as shelters and spawning territories, and floors were covered in gravel. Fish were fed cichlid flakes daily (AquaDine, Healdsburg, CA, USA). All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2011. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA.

Experimental Protocol

To examine how visual and chemosensory signals from rival males impacted the behavior and neural activation patterns in subject dominant males, I used the experimental setup shown in **Figure 2**. Experiments were conducted in a 37.85-liter tank that is divided into three equally sized compartments (16.7 × 25.3 × 30.8 cm each) by clear, acrylic barriers permanently sealed into the tank to prevent transfer of chemosensory signals. Each compartment contained a layer of gravel, an air stone, and a territory/shelter (half terracotta pot). Trials (lasting 30-40 minutes) were conducted at

the same time of day (~1-3 pm), and video recorded for later behavioral quantification. Focal dominant males were selected from community tanks the day prior to the experiment. Male dominance was visually determined by the presence of an eye bar, bright coloration, and dominance behaviors, and then later confirmed during dissection by calculating the gonadosomatic index [GSI; =(gonad mass/body mass) * 100] as a measure of reproductive investment. Focal males were placed within the middle compartment to acclimate for 24 hours, visually exposed to the community compartment on the right including a smaller dominant male and two females to reduce social isolation stress during acclimation prior to the experiment.

To observe and quantify behaviors produced by socially dominant focal males exposed to unimodal (chemosensory or visual) and multimodal (combined chemosensory

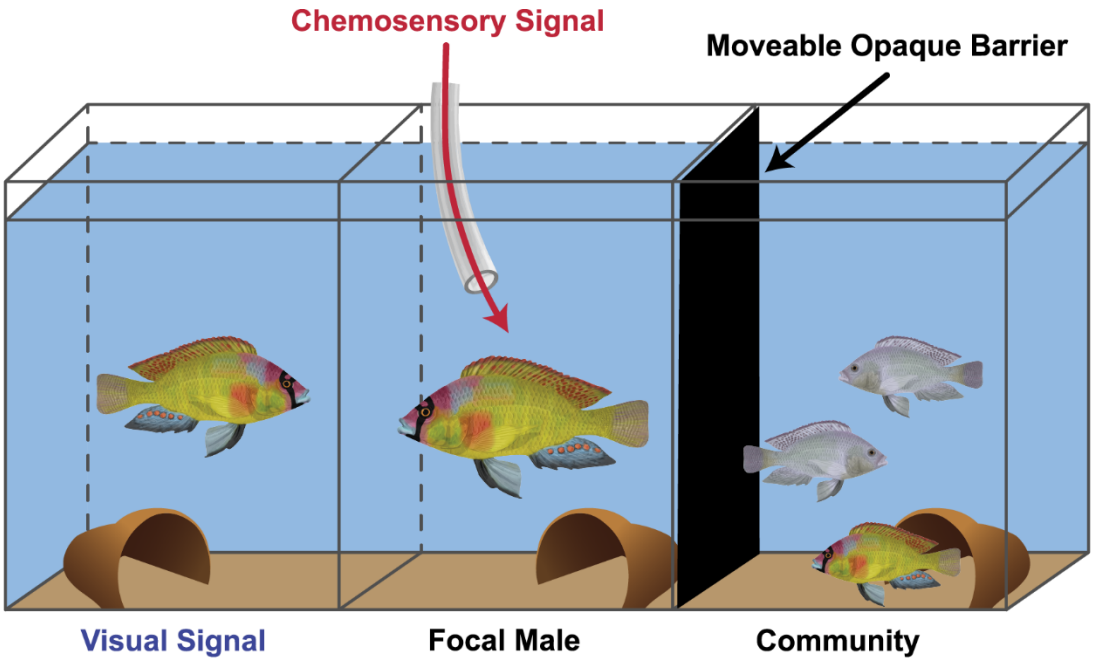

Figure 2 | Experimental setup to examine behavioral responses and neural activation patterns in dominant male *A. burtoni* exposed to multimodal and unimodal visual and chemosensory signals from other dominant males. Focal males are acclimated to the center compartment, with visual access to the community on the right. During trials, the community is blocked with an opaque barrier, visual signals (another dominant male) are presented in the left compartment and chemosensory signals are simultaneously delivered to the center compartment via a flow-controlled pump delivery system.

and visual) signals, stimuli were presented in different combinations. Visual stimuli were provided in the left compartment and consisted of either another dominant male (selected the day prior to the experiment using identical criteria to that described above for focal dominant males) that was slightly smaller than the focal male, or no fish (empty compartment control). Smaller stimulus males were used to ensure that the focal male was not defeated or forced to fall to subordinate rank during the trial. Chemosensory signals were delivered through a tube positioned in the center compartment connected to a peristaltic pump and bottle that contained either 850 mL of male-conditioned water or reverse osmosis (RO)-filtered water as a control. The male-conditioned water was made by placing three dominant males within a tank divided into three equally sized compartments. These males were allowed to behave in RO-filtered water for 4 hours, where they performed aggressive displays towards each other across the barriers. Then, 200 mL of this male-conditioned water was collected from each compartment and frozen for use in experimental trials to keep the chemosensory stimulus consistent across all trials. For use in trials, the frozen male-conditioned water was thawed and mixed with 650 ml of RO-filtered water to make the final delivery volume to 850 ml. The chemosensory delivery tube was attached to a peristaltic pump and flow rate was standardized to 0.325 L/min across treatments. Stimuli were presented in the combinations shown in **Figure 3** (visual in left compartment/chemosensory in center compartment); (1) no fish/RO-filtered water (control), (2) dominant male/RO-filtered water (vision only), (3) no fish/male-conditioned water (chemosensory only), (4) dominant male/male-conditioned water (vision + chemosensory) (n = 6 fish per condition).

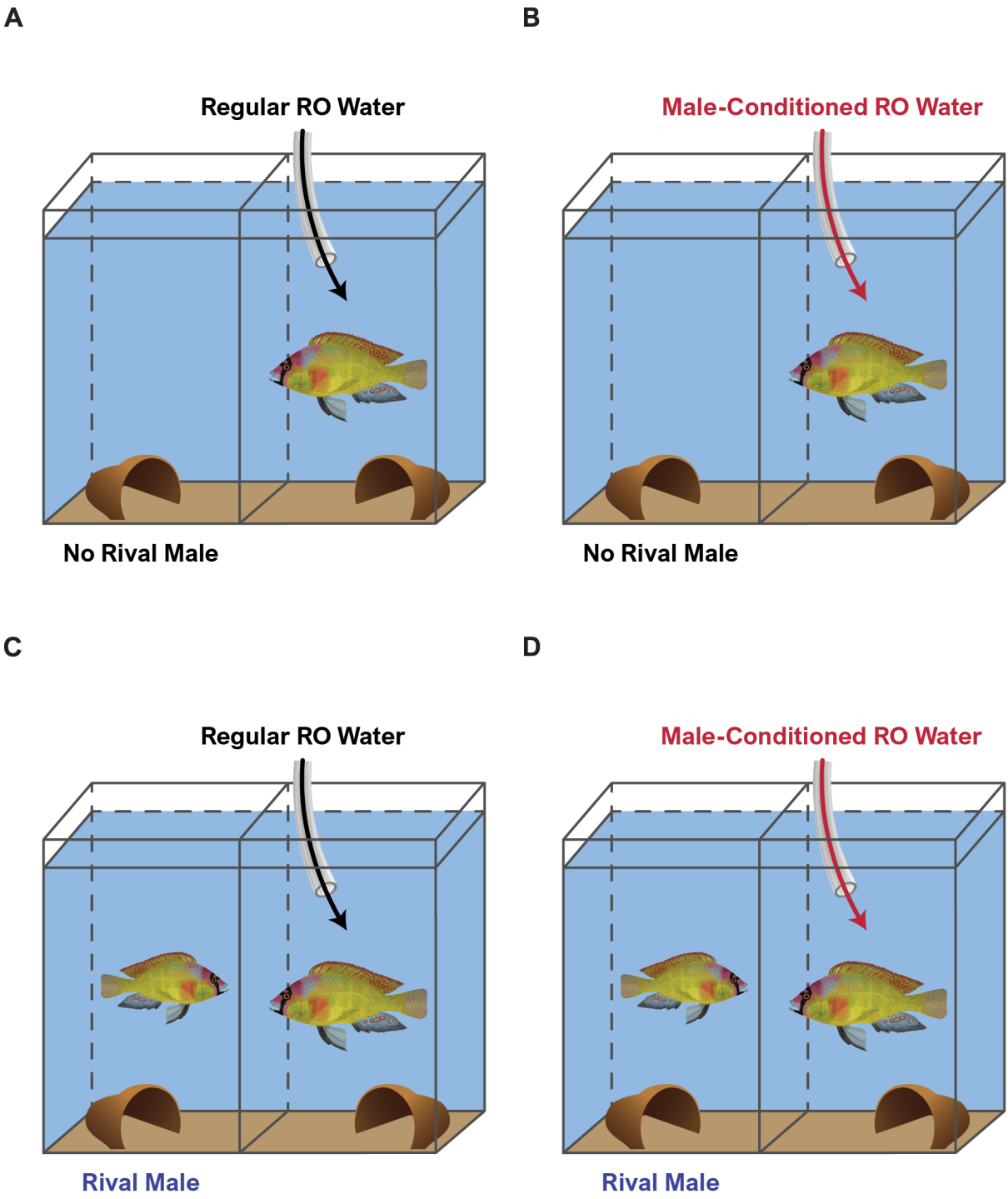

Figure 3 | Combinations of visual and chemosensory stimuli presented to focal males. **(A)** Control trial. Focal males are presented with no visual stimulus and control RO-water chemosensory stimulus. **(B)** Chemosensory Only trial. Focal males are presented with no visual stimulus and a chemosensory stimulus from other males. **(C)** Visual Only trial. Focal males are presented with a visual stimulus (dominant male) and control RO-water chemosensory stimulus. **(D)** Combined Chemosensory and Visual trial. Focal males are presented with both visual (dominant male) and chemosensory stimulus from other males.

Behavioral Quantification

Focal male behaviors and visual stimulus male behaviors (if present) during the entirety of each experiment were quantified using BORIS software (Friard & Gamba, 2016). The male behaviors quantified were categorized as non-aggressive behaviors (time spent searching, time spent in shelter, time spent near chemosensory delivery tube) and aggressive behaviors (number of lateral displays, number of frontal displays, and time spent engaged in a border fight). **Table 1** summarizes the definitions and criteria used for each behavior.

Table 1 | Description of behaviors performed by *A. burtoni* dominant males

Behavior	Classification	Description
Time In Shelter	Non-aggressive	Time spent within the terra-cotta pot placed within compartment
Time Near Chemosensory Tube	Non-aggressive	Time spent within one body length of chemosensory delivery tube
Time Spent Searching	Non-aggressive	Increased swimming speed for at least 3 seconds with at least one change in direction
Time Engaged In Border Fight	Aggressive	Confrontation between two dominant males at the site of their common border
Frontal Display	Aggressive	Threatening another dominant male by spreading operculum and fins frontally
Lateral Display	Aggressive	Threatening another dominant male by spreading operculum and fins laterally, usually associated with body vibration

Behaviors were classified as non-aggressive (behaviors performed that did not assert superiority over another dominant male) and aggressive (behaviors performed to assert superiority over another dominant male).

Tissue Preparation

To examine which regions of the brain are involved in processing visual and chemosensory information during the male-male interactions, focal males were collected immediately following each trial, anesthetized in ice-cold cichlid-system water, and sacrificed by rapid cervical transection. Total body length (TL), standard body length (SL), body mass (BM), gonad mass (GM), and GSI were recorded. Blood was collected from the caudal vein and centrifuged at 8,000 rpm for 10 minutes to isolate serum and then serum was stored at -80˚C prior to hormone assays. Brains were removed and fixed in 4% paraformaldehyde (PFA; made in 1x Phosphate Buffered Solution, PBS) at 4˚C for 24 hours, followed by rinsing in 1x PBS for 24 hours, and transferred to a 30% sucrose solution for up to 3 days before cryosectioning. Brains were then embedded in optimal cutting temperature (OCT) media, cryosectioned in the transverse plane at 20 µm, collected onto alternate charged slides, allowed to dry at room temperature for 48 hours, and stored at -80°C until staining.

In situ hybridization

In situ hybridization for *cfos*, an immediate early gene commonly uses as a proxy for neural activation, was used to quantify the differences in neural activation patterns among focal males exposed to different sensory stimuli from rival males (Butler & Maruska, 2016). Slides of cryosectioned brains were removed from -80°C storage and acclimated to room temperature. A hydrophobic barrier (Immedge pen, Vector Laboratories) was applied around the sections and allowed to dry for 30 minutes. Slides

were then incubated in 1x PBS (3 x 5 min), 4% PFA (1 x 20 min), 1x PBS (2 x 5 min), proteinase K (10 µg/ml final concentration in 50 mM Tris-HCl pH 7.5 and 5 mM EDTA pH 8.0; 1 x 10 min), 1x PBS (1 x 10 min), 4% PFA (1 x 15 min), 1x PBS (2 x 5 min), milliQ water (1 x 3 min), 0.25% pure acetic anhydride in 0.1 M triethanolamine-HCl pH 8.0 (1 x 10 min), and 1x PBS (1 x 5 min), all at room temperature. Slides were then incubated in pre-hybridization buffer (50% formamide, 5x SSC, 0.1% tween-20, 0.1% CHAPS, 5 mM EDTA, 1 mg/mL torula RNA) for 3 h at 60˚C. Slides were then incubated with DIG-labeled *cfos* riboprobe, covered with hybrislips (Life Technologies), and placed in a sealed, humidified chamber (50% formamide, 50% milliQ water) at 60-65°C. After 12-16 h, hybrislips were removed in pre-warmed 2x SSC:50% formamide with 0.1% tween-20 solution. The slides were then washed at 60-65°C with 2x SSC:formamide (2 x 30 min), 2x SSC:Maleate Buffer (MABT; 100 mM maleic acid pH 7.2, 150 mM NaCl, 0.1% tween-20; 2 x 15 min), and MABT (2 x 10 min). Slides were the incubated at room temperature in MABT (2 x 10 min) and nonspecifically blocked in MABT with 2% bovine serum albumin (BSA) (1 x 3 h). Slides were then moved into a humidified (milliQ water) chamber at 4°C and incubated in alkaline-phosphatase-conjugated anti-DIG Fab fragments (Roche; 1:5000 dilution in blocking solution). After 12-16 h, slides were removed from the cold room and rinsed in room temperature MABT (3 x 30 min) and incubated in alkaline phosphate buffer (2 x 5 min). The slides were then moved to a 37°C incubator and developed with nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) substrate (Roche) for ~3 h. Slides were then rinsed with 1x PBS (3 x 5 min), fixed with 4% PFA (1 x 10 min), and rinsed again with 1x PBS (3 x 5 min). Finally, slides were

coverslipped with aquamont media (Thermo-Scientific), dried overnight, and then edges sealed with clear nail polish for later viewing.

Quantification of Neural Activation

To quantify differences in *cfos* staining in the brain among sensory conditions, slides were visualized on a Nikon Eclipse Ni microscope and photographed with a color digital camera controlled by Nikon NIS-Elements software. Brightfield and phase contrast were used to visualize neuroanatomical markers and brain nuclei in relation to stained cells. *cfos*-positive cells were identifiable by dark purple staining inside the cell with a clear, discernible border. Neuroanatomical structures were identified using a cresyl violet stained *A. burtoni* reference brain and *A. burtoni* brain atlas. Neuroanatomical markers were used to designate the borders and rostro-caudal extent of each region to ensure consistency across animals.

The following socially-relevant regions of the brain were quantified: anterior tuberal nucleus (ATn), dorsal part of the ventral telencephalon (Vd), supracomissural nucleus of the ventral telencephalon (Vs), and anterior part of the periventricular preoptic area (nPPa). These regions were chosen because they are part of the SDMN and were shown in previous studies to be either involved in mediating aggressive behaviors or in processing visual-chemosensory information in *A. burtoni* male-female contexts (Field et al., 2018). Images were taken at the highest magnification (10x or 20x objective) that encompassed the entire area of interest. For 10x images (ATn and Vs), nuclei borders were outlined with either 50 μm × 50 μm gridlines (ATn) or 80 μm × 80 μm gridlines (Vs)

applied to each image. *cfos*-expressing cells in five randomly chosen boxes per section were counted for Vs and four randomly chosen boxes for ATn and cell density calculated by dividing the number of cells within the boxes by the total area of the boxes. For 20x images (Vd and nPPa) the same procedure was followed, except nuclei borders were overlaid with 50 μm × 50 μm grid lines and *cfos*-expressing cells were counted in three boxes. For all regions, three consecutive sections were quantified for each region and averaged together for a cell density value (#cells/μm^2) of that region for each animal. Density values were then averaged across individuals exposed to the same sensory stimulus conditions.

Statistical Analysis

Data was analyzed in JMP Pro 16.0 (SAS Institute, Cary, North Carolina). Behavior and neural activation data were compared with one-way ANOVAs. Non-aggressive and aggressive behaviors performed by both focal males and visual-stimulus males were compared using Tukey-Kramer HSD test. Data sets were checked for outliers using Grubb's test, and any outliers were excluded from statistical tests and plotted data. Statistical significance was determined at $p < 0.05$. Pearson correlations were used to test for associations between focal male behaviors and stimulus male behaviors, and between aggressive behaviors and neural activation patterns.

RESULTS

Behavioral Response to Uni- and Multimodal Visual and Chemosensory Signals

To test the how visual and chemosensory stimuli affect male-male behavior, focal males were given signals and behaviors were quantified. A total of 24 behavioral trials were conducted (N = 6 per sensory condition). Focal male body measurements (mean ± SD) for SL (mm), BM (g), and GSI for each sensory condition are as follows: control (46.2 mm ± 3.95 mm; 2.16 g ± 0.36 g; 0.59 ± 0.39), chemosensory only (44.6 mm ± 3.79 mm; 1.92 g ± 0.31 g; 0.63 ± 0.16), visual only (42.9 mm ± 2.16 mm; 1.81 g ± 0.33 g; 0.53 ± 0.21) and combined visual-chemosensory (48.1 mm ± 4.90 mm; 2.39 g ± 0.53 g; 0.49 ± 0.20). The SL (one-way ANOVA $F_{(3,20)} = 2.0318$, $p = 0.1418$), BM (one-way ANOVA $F_{(3,20)} = 2.6345$, $p = 0.0779$), and GSI (one-way ANOVA $F_{(3,20)} = 0.3553$, $p = 0.7858$) did not differ across conditions.

Focal males displayed higher levels of aggression when given both visual and chemosensory signals combined compared to unimodal conditions (**Figure 4**). Focal males given paired visual-chemosensory signals spent more time engaged in border fights (one-way ANOVA $F_{(3,20)} = 72.5630$, $p < 0.0001$) compared to those given no signal (tukey HSD, $p < 0.0001$), only a chemosensory signal (tukey HSD, $p < 0.0001$), and only a visual signal (tukey HSD, $p < 0.0001$). Males only given a visual signal engaged in border fighting more than males given no signal (tukey HSD, $p = 0.001$) and those given only a (tukey HSD, $p = 0.001$) chemosensory signal. Focal males given both visual and chemosensory signals performed more frontal displays (one-way ANOVA $F_{(3,20)} = 8.8944$, $p < 0.0001$) than males given no signal (tukey HSD, $p = 0.001$), only a chemosensory

signal (tukey HSD, $p = 0.001$), and only a visual signal (tukey HSD, $p = 0.001$). Focal males given both visual and chemosensory signals performed more lateral displays (one-way ANOVA $F_{(3,20)} = 72.5630$, $p < 0.0001$) than focal males given no signal (tukey HSD, $p = 0.00039$) or only a chemosensory signal (tukey HSD, $p = 0.0039$). The duration of nonaggressive behaviors was not significantly influenced by the presence or absence of visual and chemosensory signals (**Figure 5**). In summary, focal males only perform aggressive behaviors when visually exposed to another male and are more aggressive when that visual signal is coupled with a chemosensory signal produced by another dominant male.

Behavioral Reponses between Focal Male and Stimulus Male

To examine the aggressive behaviors performed between the stimulus male (in visual trials) and the focal male over time, I used raster plots to visualize their reciprocal interactions (**Figure 6**). These raster plots also illustrate the increased aggressive behaviors in visual-chemosensory trials that often lasted the entire trial period. Regardless of the presence or absence of a chemosensory signal, there was a positive correlation between the aggressive displays performed by the visual signal males and those performed by the focal male, illustrating the reciprocal nature of these territorial interactions (**Figure 7**).

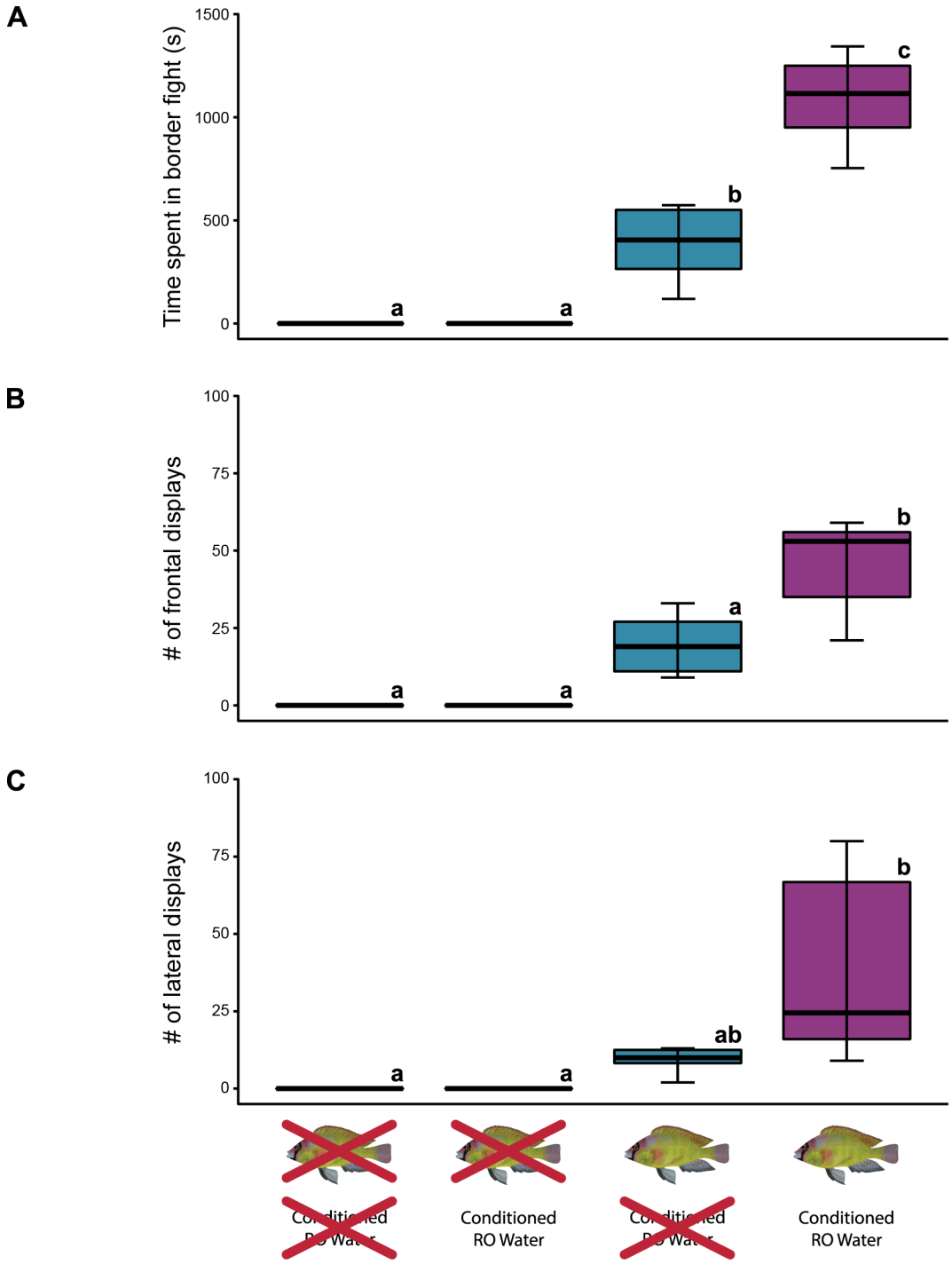

Figure 4 | Visual signals from opposing males are required for aggressive behaviors but combined visual and chemosensory signals dramatically increase behaviors in male-male territorial contexts. Focal males exposed to a combination of visual and chemosensory stimuli **(A)** spend more time engaged in a border fight with the visual signal male, **(B)** produce more frontal displays, and **(C)** produce more lateral displays through the 30-minute behavioral trial compared to the visual only trial. The focal males not exposed to an opposing dominant male, control, and chemosensory trials, produced no aggressive behaviors. Different letters indicate statistical differences (Tukey's HSD, $p < 0.05$, $n = 6$). Boxplots are used to represent data: solid line in box = mean; box extends to the furthest data points within the 25th and 75th percentile; whiskers extend to the 10th and 90th percentile.

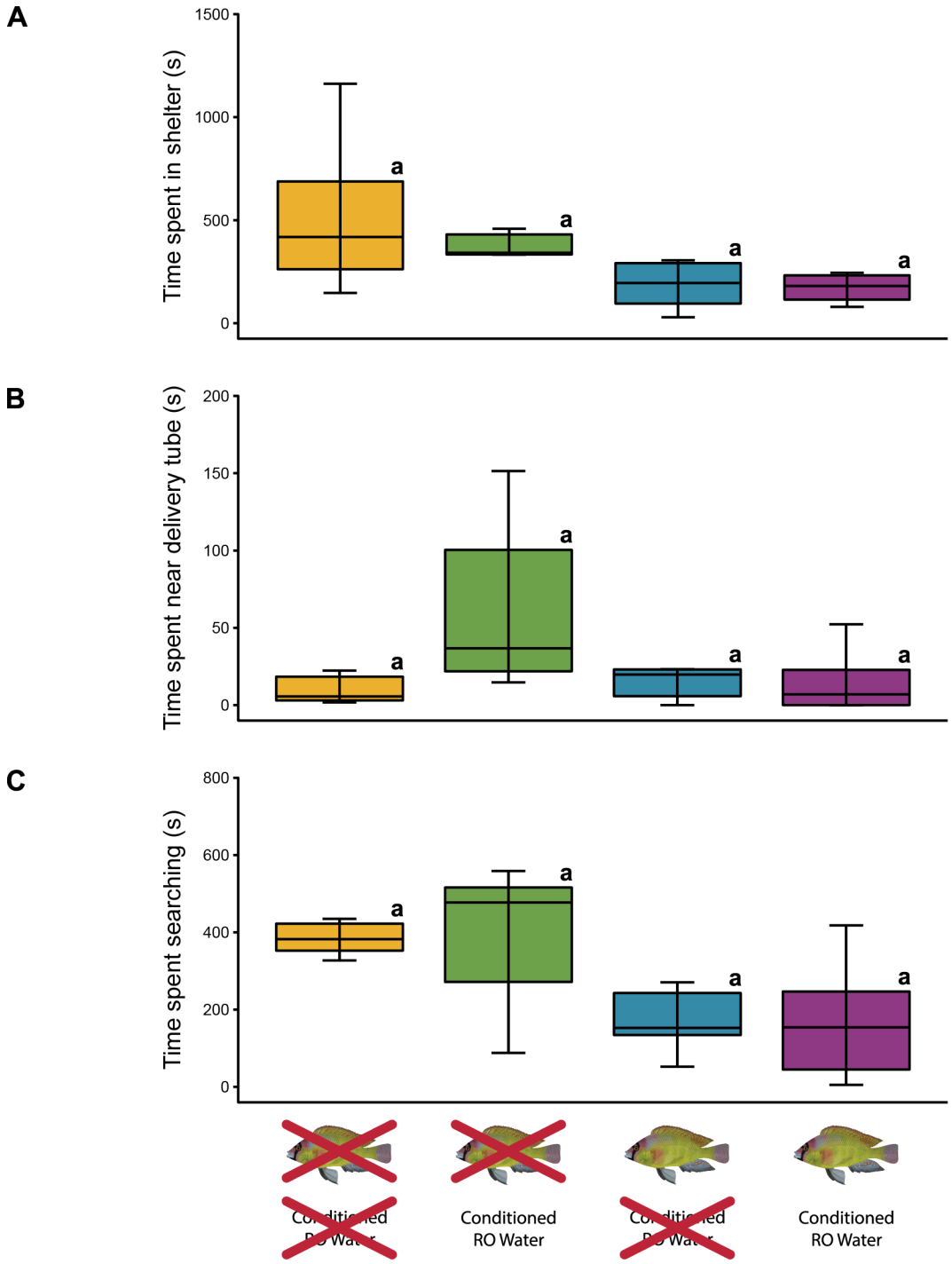

Figure 5 | Visual and chemosensory signals from opposing dominant males do not influence nonaggressive behaviors in focal males. The duration of nonaggressive behaviors [**(A)** time spent in shelter, **(B)** time spent near chemosensory delivery tube, and **(C)** time spent = searching)] displayed by the focal male, through the 30-minute behavioral trial was not significantly different among behavioral groups. Different letters indicate statistical differences (Tukey's HSD, $p < 0.05$, $n = 6$). See Figure 4 caption for box plot format.

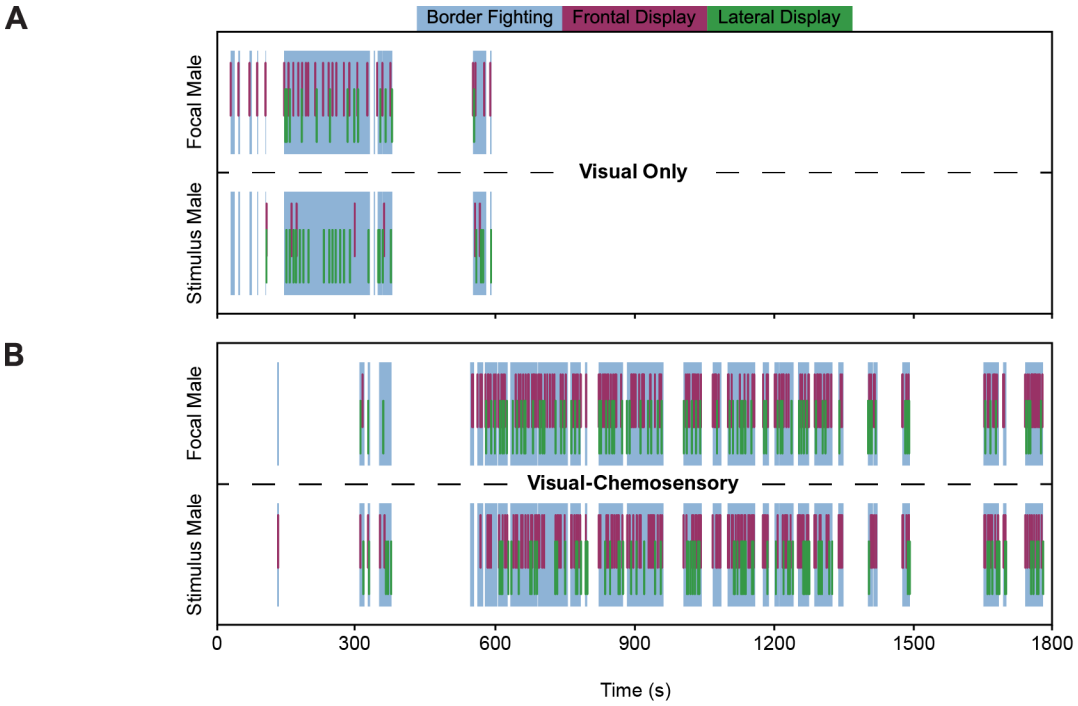

Figure 6 | Representative examples of aggressive displays by the stimulus and focal males over the time course of a single trial. Raster plots showing aggressive behavior from the **(A)** Visual Only trial and the **(B)** Visual-chemosensory trial. Frontal displays (blue) and lateral displays (green) produced by the focal male (top) and visual stimulus male (bottom) are shown at the time they were produced during the 30-minute behavioral trial.

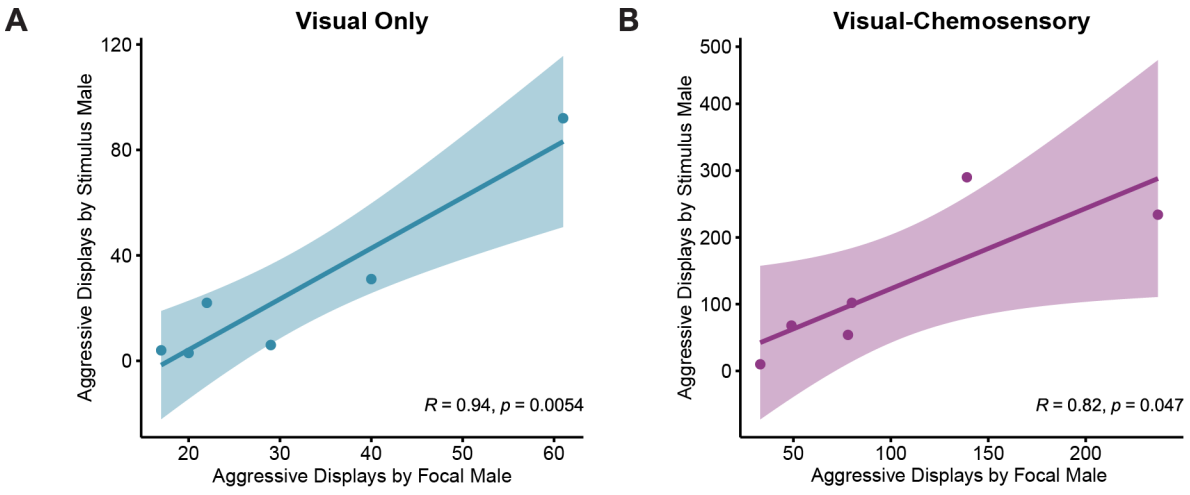

Figure 7 | Aggressive displays are positively correlated between the visual stimulus male and the focal male in both **(A)** visual only and **(B)** combined visual-chemosensory trials.

Neural Activation

Figure 8 shows a representative low magnification cresyl violet-stained transverse sections from *A. burtoni* with locations of relevant regions quantified for neural activation (measured as *cfos* cell density) in this study. Focal males given no signal or only a chemosensory signal had lower levels of *cfos* expression in measured areas compared to focal males given only a visual signal or given both a visual and chemosensory signal (**Figure 9-10**). Due to time constraints, I was only able to quantify activation in 1 fish per sensory treatment group, so statistical comparisons cannot yet be performed. Additional brain activation data will be analyzed in the future to add to this data set.

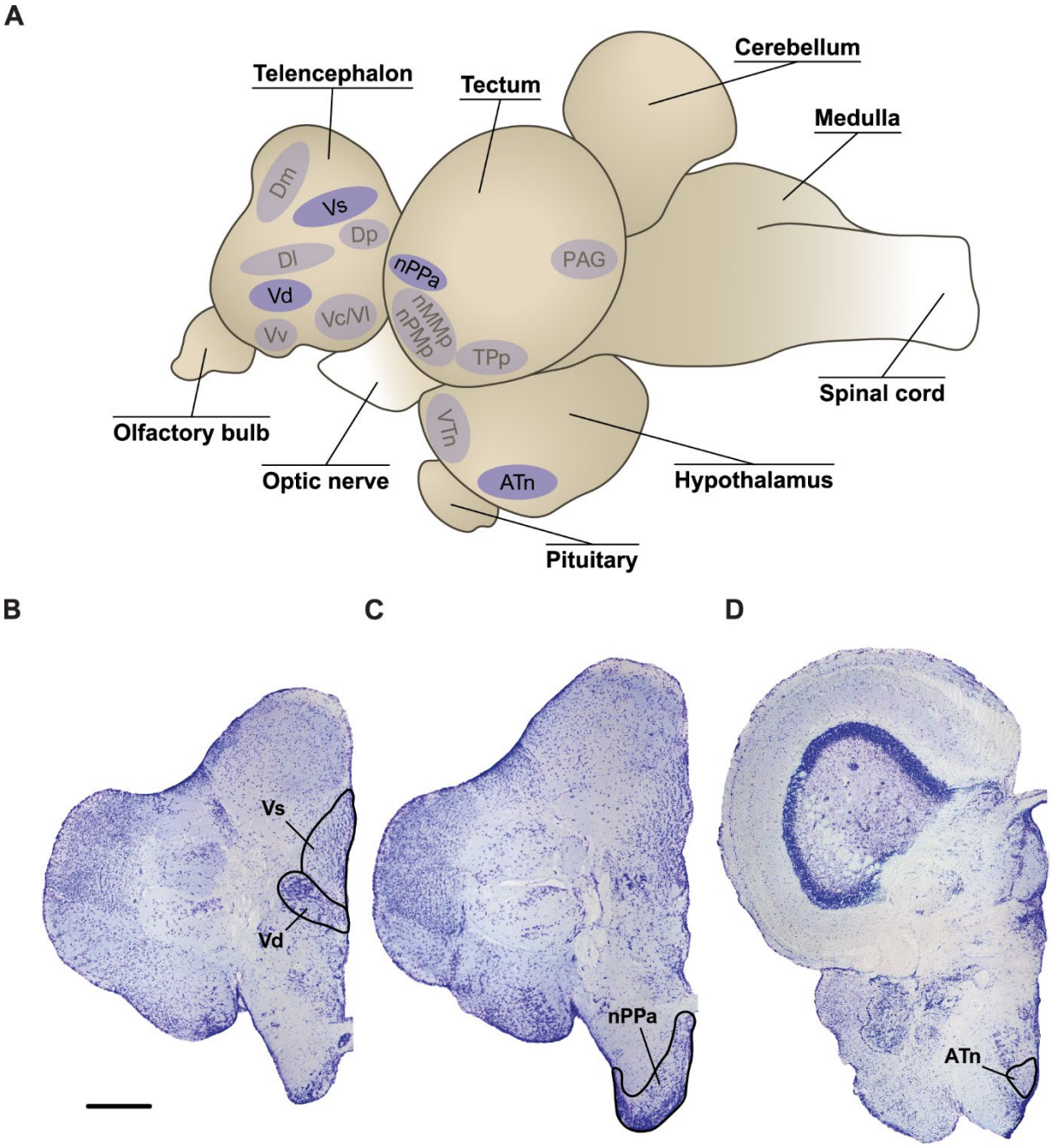

Figure 8 | *A. burtoni* brain regions used to quantify neural activation patterns. **(A)** Social-decision making network (SDMN) of A. burtoni. Representative cresyl-violet stained transverse sections are shown from rostral **(B)** to caudal **(D)** and nuclei quantified in this study are outlined and labeled. Scale bar = 250 μm. Abbreviations: ATn, anterior tuberal nucleus; Dl, lateral zone of the dorsal telencephalon; Dm, medial part of the dorsal telencephalon; Dp, posterior part of the dorsal telencephalon; nPPa, parvocellular preoptic nucleus, anterior part; nMMp, magnocellular preoptic nucleus, magnocellular division; nPMp, magnocellular preoptic nucleus, parvocellular division; PAG, periaqueductal gray; TPp, periventricular nucleus of the posterior tuberculum; Vc, central part of the ventral telencephalon; Vd, dorsal part of the ventral telencephalon; Vl, lateral part of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; VTn, ventral tuberal nucleus; Vv, ventral part of the ventral telencephalon.

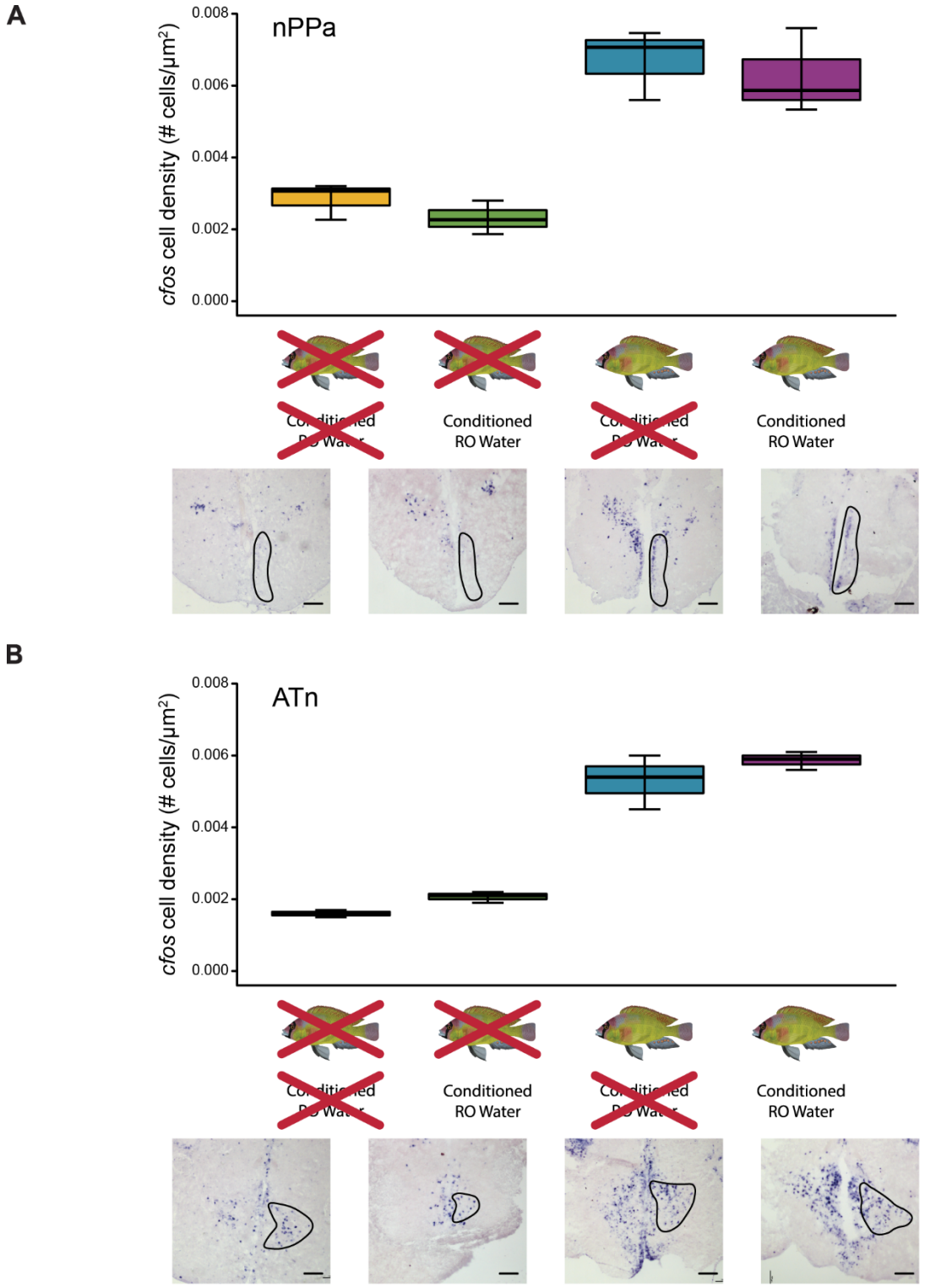

Figure 9 | Unimodal and multimodal visual and chemosensory signals from other dominant males elicit distinct neural activation patterns in dorsal telencephalic and hypothalamic brain regions of dominant *A. burtoni* males. There is greater cfos expression in **(A)** ATn and **(B)** nPPa when a visual signal is present, regardless of whether a chemosensory signal is present or not. Photos show representative examples of *cfos* staining in each region for conditions. Outlines demonstrate approximate quantified area for each region. Scale bars represent 100 µm.

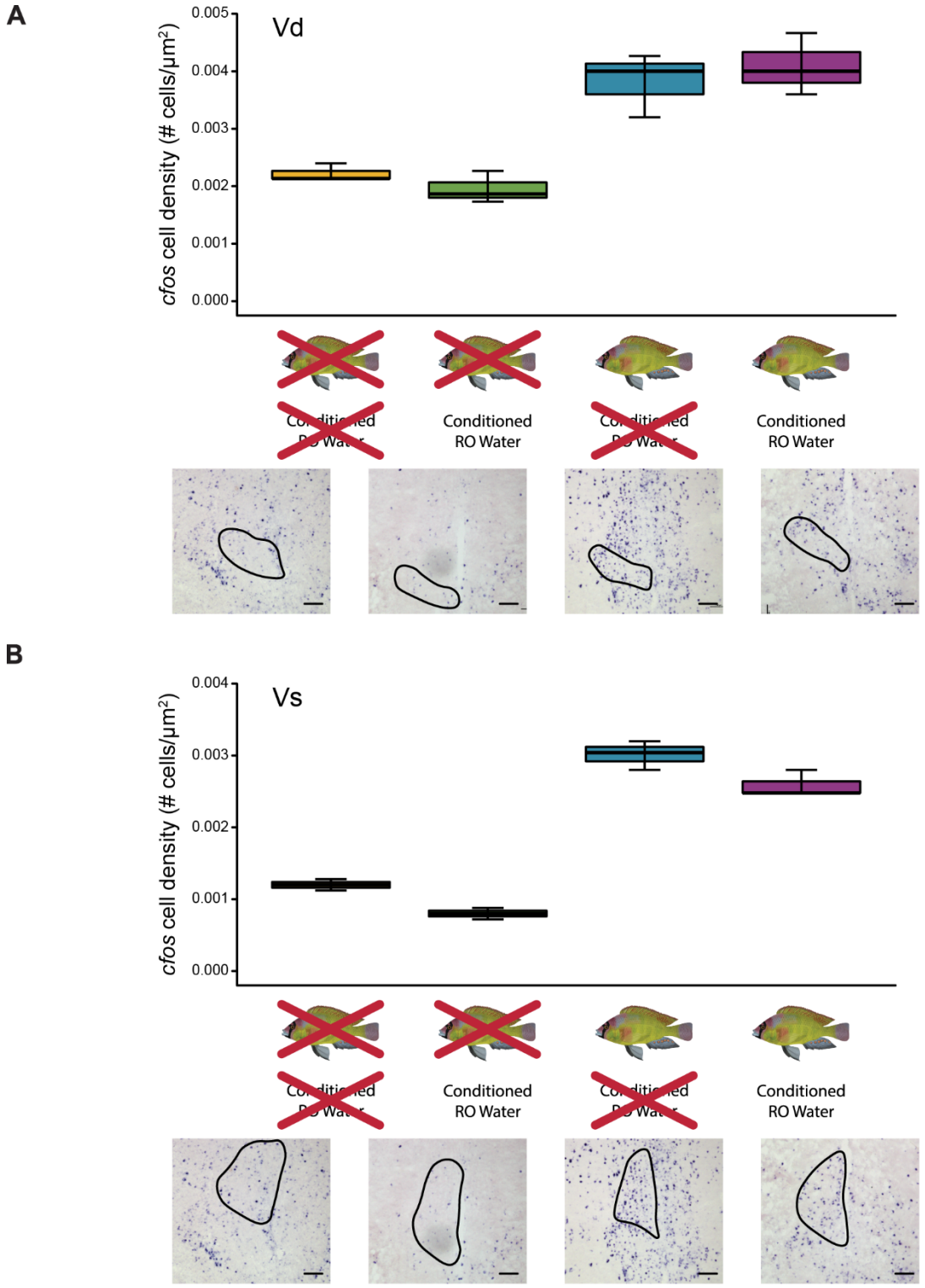

Figure 10 | Unimodal and multimodal visual and chemosensory signals from other dominant males elicit distinct neural activation patterns in ventral telencephalic brain regions of dominant *A. burtoni* males. There is greater *cfos* expression in **(A)** Vd and **(B)** Vs when a visual signal is present, regardless of whether a chemosensory signal is present or not. Photos show representative examples of *cfos* staining in each region for conditions. Outlines demonstrate approximate quantified area for each region. Scale bars represent 100 µm.

DISCUSSION

I investigated behavioral responses and associated neural activation patterns of dominant *A. burtoni* males to uni- and multimodal visual and chemosensory signals from another dominant male. My results show that males need visual signals from another male to engage in aggressive behaviors such as frontal displays and lateral displays, and that chemosensory signals alone are not sufficient to motivate these aggressive behaviors. However, the number of aggressive behaviors was greater when males were simultaneously exposed to visual and chemosensory signals from another male, compared to either sensory signal alone. This indicates that chemical signals motivate aggressive displays in male-male contexts, providing additional information not obtained from visual signals alone. Using the immediate early gene *cfos* as a proxy for neural activation, my preliminary data also revealed that decision processing regions show differential activation when dominant males are exposed to visual and chemosensory signals together compared to exposure of either sensory signal alone. These results demonstrate that chemosensory signals released from dominant males in aggressive contests provide additional important information that can be used to decide territorial disputes, with important consequences for reproductive fitness and survival.

Behavioral Responses to Unimodal and Multimodal Signals

Animals that exist in dominance hierarchies must actively and constantly assess their surrounding environment to receive important information such as motivation and social status from sending animals (Simões et al., 2015). My study showed that dominant

A. burtoni males require visual signals from rival dominant males to initiate aggressive behaviors, further escalating when paired sensory signals were given. Since unimodal visual and chemosensory signals elicited different behavioral responses compared to the combined visual-chemosensory multimodal signal, it is reasonable to say that visual-chemosensory multimodal signals from rival dominant *A. burtoni* males conveys different information to the focal male (i.e., are non-redundant). When only visual signals are present, the focal male produces aggressive behaviors to a lesser extent than the combined visual-chemosensory signals. In a previous study with the Mozambique tilapia *Oreochromis mossambicus,* dominant males showed higher levels of aggressive behaviors as urination frequency increased, and dominant males stopped releasing urine once the rival male showed submissive behaviors (Barata et al., 2007). *A. burtoni* dominant males dramatically increased the number of courtship behaviors when given both visual and chemosensory signals from gravid females compared to only being given a visual signal (Field et al., 2018). Males can also distinguish between gravid nonovulated females from ovulated females (Butler et al., 2019) likely based on chemosensory signals. It is possible that visual signals act as a "bare-minimum" informative signal with regard to social status, as *A. burtoni* dominant males are phenotypically distinct from less threatening subordinate males, and chemical signals act as a type of reinforcing signal, providing enough information about the rival male to increase aggression to protect their dominant status.

Multimodal signaling also allows for reduced habituation of individual signals, and allows for continued information transmission in conditions where one signaling modality

may be masked or eliminated (e.g., vision reduced in turbid conditions) (Todt & Fiebelkorn, 1980). Within my study, this can be seen by comparing the occurrences of the quantified aggressive behaviors over the course of the trial. Focal males given only a visual signal spent less time engaged in border fights towards the end of the trial compared to focal males given both a visual and chemical signal. In a previous study, dominant *A. burtoni* males also performed more aggressive behaviors when exposed both visually and chemically to rival males compared to dominant males only exposed visually (Maruska & Fernald, 2012). This suggests that the addition of the chemical signal motivates the focal male to continue performing aggressive behaviors.

All visual stimulus males throughout the experiment were only visually exposed to the focal male, yet the visual stimulus males perform approximately the same number of behaviors as the focal male regardless of the presence of a chemical signal. A previous study in *A. burtoni* showed that visual stimuli alone from a larger dominant male can suppress aggressive behaviors of smaller dominant males (Chen & Fernald, 2011). The discrepancy between this study and mine could be explained by size differences between the visual stimulus male and the focal dominant male, as my study used similarly-sized males where the previous study used visual stimulus males 4-times the size of the focal male. The difference in behaviors based on size could be due to the accuracy of dominant males to assess the size of their opponent using only visual information. Similarly sized males may perform aggressive behaviors at a similar rate with only visual exposure as they have a similar chance of winning the fight. Within the combined visual-chemosensory trials, however, the focal male was typically the one to initiate each border fight. This

suggests that a dominant male does not need a chemical signal to reciprocate aggression, but it is the chemical signal that enhances aggression.

Neural Activation

Using *cfos* as a marker for neural activation, I quantified aggression-relevant regional activity in males exposed to visual and chemosensory information from other males in territorial contexts. The social decision-making network (SDMN) is a collection of highly conserved brain nuclei responsible for regulating and initiating responses to social stimuli (Newman, 1999; O'Connell & Hofmann, 2011). While not enough neural activation data was collected here for statistically comparisons, these data provide a preliminary basis for the understanding of how visual-chemosensory stimuli are integrated within the brain to produce aggressive behaviors. Current data shows increased activation in the measured SDMN regions following visual signals given either alone or paired with a chemical signal, suggesting that the nPPa, ATn, Vd, and Vs are all involved in mediating aggressive behavior and processing visual-chemosensory information.

The nPPA (putative homolog of the paraventricular region of the preoptic area in mammals) is a preoptic area sub-region that is extensively studied for its role in social behaviors such as parental care, male copulatory behavior, and general reproductively-relevant behaviors across many taxa. It is also known as a sensory integration center, which results in neuroendocrine and motor responses in a variety of social contexts such as aggression and reproduction (Forlano & Bass, 2011). In a reproductive context, male *A. burtoni* also showed increased neural activation in this region when receiving visual

and chemosensory information from females (Field et al., 2018). Thus, it appears the preoptic area is an important visual-chemosensory processing center in both male-female reproductive and male-male aggressive contexts.

The ATn (putative homolog of the ventromedial hypothalamus (VMH) in mammals), is involved in mediating aggressive behaviors across vertebrates. In the mammalian VMH, *cfos* expression is elevated following aggressive interactions (Kollack-Walker & Newman, 1995; Lin et al., 2011). In male *A. burtoni*, the ATn is important in the processing of mechanosensory signals from lateral line stimulation following aggressive interactions (Butler & Maruska, 2016) as well as the transition between subordinate and dominant social states (Maruska et al., 2013). Thus, my activation results suggest that the ATn also receives visual and chemosensory information during male-male interactions.

Due to its connection with reward centers in the ventral tegmental area of the midbrain, the Vd (putative homolog of the nucleus accumbens in mammals) acts to enforce or avoid behaviors based on various stimuli (Ikemoto & Panksepp, 1999). These nuclei are important telencephalon regions for social decisions, and the increased complexity of neurons in these areas of dominant males may aid in their acquisition of the physiology and behaviors required for dominance status. Stimulation of the Vs (putative homolog of the extended amygdala in mammals) leads to an increase in both aggressive and reproductive behaviors in fishes (Demski & Knigge, 1971; Satou et al., 1984). The Vs is also known to process multimodal sensory information in *A. burtoni* and other fishes, and it seems it is also involved in visual-chemosensory processing in aggressive contexts shown here.

Overall, these data provide the basis for identifying socially-relevant visual-chemosensory processing in regions of the SDMN following male-male territorial contests. Further investigation for statistical significance and remaining regions of the SDMN is needed to understand how aggressive-relevant visual-chemosensory information is processed in the brain, and how that processing leads to adaptive behaviors. Further quantification in all SDMN regions will allow for the examination of co-activation networks, which will reveal patterns of brain-wide activity governing aggressive behaviors in response to unimodal and multimodal visual-chemosensory inputs.

SIGNIFICANCE

Studying multisensory processing is important because all animals live in a multimodal world and many communicate in more than one sensory channel. However, how this sensory information is translated into decisions is not well understood because most studies in animal communication focus on only one sense at a time. The data I collected provides the basis for identification of nuclei responsible for processing visual and chemosensory information in male-male territorial contexts. This work provides novel information on the importance of chemosensory-visual signaling as a potential substrate for sexual selection, speciation, and factors that may be driving evolutionary changes in fishes, the largest and most diverse group of vertebrates.

CONCLUSIONS

My study investigated the role of uni- and multimodal visual-chemosensory signaling in mediating aggressive behaviors in the cichlid fish *Astatotilapia burtoni*. Using behavioral analyses, my data showed that visual signals are required for male-male aggressive interactions, and that paired visual-chemosensory signals dramatically increase aggression in territorial contexts. This demonstrates that chemosensory signals released from fighting males provide important additional information not conveyed by vision, potentially on dominance status, that helps modulate aggressive interactions during territorial disputes. Preliminary neural activation data provides a basis for understanding the underlying processes governing the production of aggressive behaviors in response to visual-chemosensory signaling.

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