

## RESEARCH ARTICLE

# Context-dependent chemosensory signaling, aggression and neural activation patterns in gravid female African cichlid fish

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## ABSTRACT

Social animals must constantly assess their environment to make appropriate behavioral decisions. The use of various sensory modalities is imperative in this process and it is hypothesized that the highly conserved brain nuclei comprising the social decision-making network (SDMN) integrates social information with an animal's internal state to elicit behavioral responses. Here, we used the highly social African cichlid fish, *Astatotilapia burtoni*, to investigate whether reproductively receptive (gravid) females show contextual chemosensory signaling, social behaviors and neural activation patterns within the SDMN. We exposed gravid females to different social contexts: (1) dominant male (inter-sexual reproductive); (2) mouth brooding (non-receptive) female; (3) gravid female (intra-sexual aggressive); (4) juvenile fish (low social salience); and (5) empty compartment (control). By injecting females with a blue dye to visualize urine pulses, we found that gravid females show context-dependent urination, exhibiting higher urination rates in the presence of dominant males (reproductive context) and mouth brooding females (aggressive contexts). Further, gravid females show contextual aggression with increased aggressive displays toward mouth brooding females compared with other gravid females. Using *in situ* hybridization to quantify cells expressing the immediate early gene *cfos* as a measure of neural activation, we also show that certain regions of the SDMN in gravid females are differentially activated after exposure to high compared with low social salience contexts. Coupled with previous reports, these results demonstrate true chemosensory communication in both sexes of a single fish species, as well as reveal the neural substrates mediating intra- and inter-sexual social behaviors in females.

**KEY WORDS:** *Astatotilapia burtoni*, Communication, Olfaction, Social decision-making network, Teleost, Urine

## INTRODUCTION

Social animals must constantly integrate their own internal state with their environment to make appropriate behavioral decisions, such as those involved in mating, parental care and aggression. Most animals live in a multimodal world, making the signaling, reception and integration of information across several senses, such as vision, audition, mechanoreception, somatosensation and chemoreception, crucial for their ability to respond appropriately to relevant stimuli (Bradbury and Vehrencamp, 2000). For example, whereas visual signals can be used to communicate over relatively long distances,

chemical signaling is often employed while in close proximity to provide additional information on an individual's sex and reproductive fitness (Chamero et al., 2012; Rollmann et al., 1999; Sorensen, 1992). While it is known that detection of these signals is crucial for animals to make social decisions, most studies address animal communication only from the perspective of the receiver or the sender. To gain better insight into animal communication, it is important that social signaling be understood from the perspective of both signalers and receivers in different social contexts.

Chemoreception is a phylogenetically old sense among vertebrates, which is imperative for survival and reproduction across diverse animal taxa from insects to mammals (Isogai et al., 2011; Kotschal, 2000). In fishes, chemoreception is used for food detection, predator avoidance, migration, kin recognition and reproduction, with relevant compounds detected by the olfactory and gustatory systems (Hara, 1994; Kotschal, 2000). The use of urine as a means of chemical communication is widely employed across fish species, conveying information on sex, reproductive fitness, metabolic state and social status (Giaquinto and Volpato, 1997; Stacey, 2011). Whether urine is actively or passively released in inter- and intra-sexual social contexts, however, varies across species (Almeida et al., 2005; Appelt and Sorensen, 2007; Keller-Costa et al., 2015). Despite the importance of chemical signaling in fishes, relatively little is known about how most species use chemosensory communication, with only a few of the more than 30,000 extant species investigated thus far. Further, few studies examine chemical signaling in inter- and intra-sexual interactions in both sexes of a single species, which is important for understanding the selective pressures that have shaped the evolution of communication systems.

Cichlid fishes are excellent models to examine the mechanisms of communication from both a signaler and a receiver perspective. Their plastic behavioral repertoire, ease of social manipulation and recent genome sequencing make cichlids, and *Astatotilapia burtoni* in particular, an important model for studying sexual selection and the behavioral and molecular basis of complex social communication (Brawand et al., 2014; Maruska and Fernald, 2014; Van Straaden and Smith, 2011). Dominant *A. burtoni* males engage in aggressive displays to defend spawning territories from rival males and actively court females, enticing them into their territories to spawn by performing elaborate courtship displays, consisting of body quivers, tail waggles, chases and leading of females into a spawning territory (Fernald, 1977). Once a female is gravid (full of ripe eggs) and reproductively receptive, she will choose and follow a dominant male into his territory, spawn and then brood the developing young in her mouth for about two weeks (mouth brooding) (Renn et al., 2009). *Astatotilapia burtoni* is a very visual fish but the use of non-visual modalities such as chemosensory communication would be advantageous because their natural habitat in the shallow shore pools of Lake Tanganyika, Africa, is dynamic with variable visual conditions (Fernald, 1977).

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In fact, recent research shows that dominant *A. burtoni* males simultaneously perform visual and acoustic displays towards females (Maruska et al., 2012), as well as active chemosensory signaling via urination (Maruska and Fernald, 2012). Further, *A. burtoni* males increase reproductive behaviors when exposed to water that housed gravid females, suggesting that compounds released by females are used in reproduction (Crapon de Caprona, 1980). How *A. burtoni* females might alter the release of relevant chemical cues in different social contexts, however, remains unexplored.

Across vertebrates, information received via multiple sensory channels must be integrated in the brain to elicit suitable behavioral responses. Processing of this social information is mediated by conserved, interconnected brain nuclei of the social behavior network (Goodson, 2005; Newman, 1999). This network of brain regions may also interact with regions of the mesolimbic reward system to form a larger conserved social decision-making network (SDMN) (O'Connell and Hofmann, 2011). The acceptance of the SDMN as a pan-vertebrate model, however, requires experimental testing of how these brain regions are involved in or respond to varying social contexts in a wide range of diverse vertebrate taxa. Understanding neural activation patterns associated with specific behaviors in different social contexts will help link sensory input and behavioral output, as well as provide insight into the evolution of social behavior across vertebrates.

The goals of this study were twofold. First, we tested the hypothesis that gravid *A. burtoni* females show context-dependent chemosensory signaling and social behaviors, such as aggressive displays, in different social environments. Second, to better understand the role of the SDMN in context-dependent behaviors, we used *in situ* hybridization for the immediate early gene (IEG) *cfos* to examine neural activation patterns of gravid females exposed to different social contexts. This allowed us to identify and localize regions of the female brain that are activated in contexts that differ in social valence (e.g. inter-sexual, intra-sexual, low social salience) and during the display of context-dependent behaviors such as chemosensory signaling and aggression.

## MATERIALS AND METHODS

### Experimental animals

Adult *Astatotilapia burtoni* (Günther, 1894) from a wild-caught stock were kept in aquaria under water and lighting conditions that mimic their natural habitat in Lake Tanganyika, Africa (28°C; pH 8.0; 12 h:12 h light:dark cycle). These fish were bred in laboratories since original collection in the 1970s and exhibit behaviors similar to those in wild populations (Fernald, 1977). Aquaria contained gravel-covered floors and half terracotta pots to serve as shelters and spawning territories. Fish were fed cichlid flakes (AquaDine, Healdsburg, CA, USA) daily and supplemented with brine shrimp twice a week. All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2011. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA.

### Experimental protocol

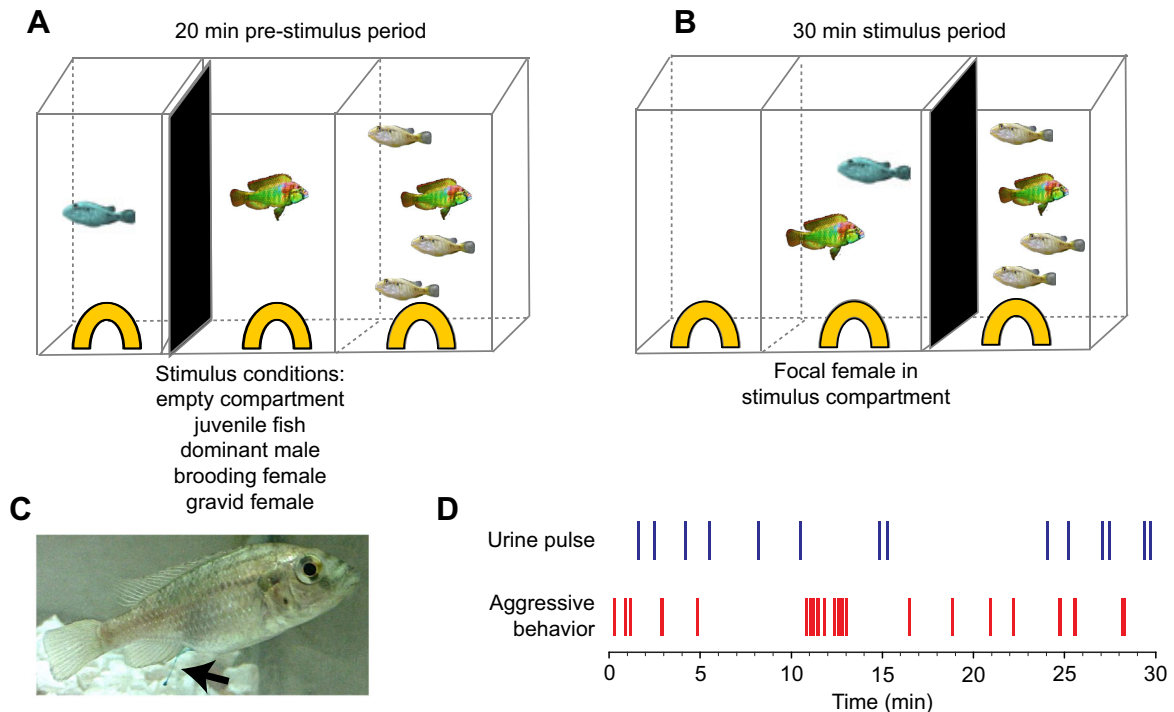
To test for context-specific chemosensory signaling, behavior and brain activation patterns of gravid females, experiments were conducted in 37.85 litre aquaria partitioned into three equal-sized compartments (26×16.5×33 cm for each) (Fig. 1A,B). Each compartment contained a half terracotta pot to serve as a territory/

shelter, and compartments were separated by clear acrylic barriers that were permanently sealed into the walls and floor of the tank to ensure no transfer of water or odorants between adjacent sections. The seals were verified by placing colored dye into the water of a single compartment and observing no transfer of dyed water between compartments. All experimental compartments were drained and refilled between experiments to prevent cross-experiment contamination. Aquaria contained white gravel and a white backdrop to more effectively visualize the blue dye concentrated in the urine as it was released from the fish (see below). Prior to each experimental trial, one of four different context-specific stimulus fish (i.e. adult dominant male, gravid female, brooding female or juveniles) from community breeding tanks of mixed multiple broods was selected and placed in experimental compartments and allowed to acclimate for 24–48 h in the center compartment while visually exposed to a community consisting of one dominant male and three females in the right adjacent compartment. During acclimation, a black, removable barrier visually blocked the left empty experimental compartment until the day of the trial (Fig. 1A,B).

### Dye injection and experimental procedure

Focal gravid females were selected from community tanks the day before experimental trials prior to feeding, and chosen based on an extended abdomen indicating the presence of large ova. Reproductive potential was established after experiments by measuring the gonadosomatic index [GSI=(gonad mass/body mass)×100], and only focal gravid females with GSI ≥7.0 were used in analyses. On the evening before the experimental trial (16:30 h–18:00 h), focal females were injected with 15–20 µl of Patent Blue Violet (PBV) dye (25 mg ml<sup>-1</sup> in 0.9% saline, Sigma–Aldrich Inc., St Louis, MO, USA) into the dorsal musculature. PBV is an innocuous dye that concentrates in the urinary bladder, producing easily visible urine pulses, and was used in previous studies to quantify fish urination rates (Appelt and Sorensen, 2007; Barata et al., 2008, 2007; Maruska and Fernald, 2012) (Fig. 1C). After injection, females were placed in a bucket of community tank water for 5 min to allow excess dye to leach out of the skin, and then acclimated overnight in the left compartment of the experimental tank that was visually separated from the center compartment by a black barrier (Fig. 1A).

The following morning, behavioral trials began prior to feeding between 08:20 h and 09:00 h. Each trial consisted of a 20 min pre-stimulus period with the focal gravid female still in isolation to obtain baseline urination data for that individual fish. Immediately after the pre-stimulus period, the focal female was quickly moved via dip-net to the center compartment for a 30 min stimulus period. The black barrier was simultaneously moved from the left side to the right side of the center compartment to expose the now-empty, left compartment and block visualization of the adjacent community on the right side during the stimulus period (Fig. 1B). To test the hypothesis that gravid females show context-dependent urination and behavior, focal females were exposed to the following different social contexts: dominant males (inter-sexual) ( $N=14$ ), gravid females and brooding females (intra-sexual) ( $N=10$  gravid,  $N=10$  brooding), juveniles (low social salience) ( $N=11$ ) and empty compartment ( $N=10$ ). Each focal gravid female was only used once, such that 55 gravid females [standard length (SL) 36.08 ±1.88 mm (mean±s.d.)] were used for this experiment. All adult stimulus fish were appropriately size-matched to the focal female and only juveniles (SL ≤9.0 mm) showing no coloration or reproductive behaviors were used as stimulus fish. All brooding



**Fig. 1. Experimental tank setup and example of urine pulse release behavior from gravid female *Astatotilapia burtoni*.** (A) To examine context-dependent chemosensory signaling, aggression and neural activation, focal gravid females (blue fish) were injected with Patent Blue Violet dye in the evening prior to experiments, and isolated in the left compartment overnight behind a removable opaque black barrier. Stimulus fish (dominant male condition is shown and all stimulus conditions are listed below) were acclimated in the center compartment with visual exposure to a community in the right compartment for 2 days prior to experiments. In the morning of the experimental trial, a 20 min pre-stimulus period was filmed and viewed live by an observer to obtain baseline behaviors and urination rates. (B) Immediately following the pre-stimulus period, the focal female was moved into the center compartment with the stimulus fish. The opaque barrier was simultaneously moved from the left to the right side, visually blocking the community on the right and exposing an empty compartment on the left. A 30 min stimulus period was filmed and viewed live to quantify behaviors and urination rates. (C) Example urine pulse (arrow) released from focal gravid female. (D) Example of the temporal sequence of urine release and aggressive behaviors exhibited by a focal gravid female exposed to a brooding female. Urine release never occurred coincidentally with aggressive behaviors but rather at times of pausing or slow swimming. Each vertical mark represents a single urine pulse or aggressive behavior (bites and lateral displays) during the 30 min stimulus period.

females (SL  $36.33 \pm 1.73$  mm) used as stimulus fish were 4–6 days into the 12 day brooding cycle and carrying developing fry. All stimulus males were deemed dominant by exhibiting bright coloration and showing behaviors typical of dominance (i.e. defending a territory, courting females) (SL  $43.0 \pm 3.69$  mm) and had a GSI  $\geq 0.70$ . All stimulus and focal gravid females had a GSI within 0.40 of one another in each experiment to ensure matched reproductive state. All trials were filmed and viewed live by an observer to quantify behaviors of focal gravid females. Behaviors were classified into two categories: chemosensory signals, and other social behaviors. Chemosensory signals included the number of urine pulses, whereas other social behaviors consisted of aggressive behaviors, including bites and lateral displays. Bites occurred when one fish opened its mouth and bit another fish, typically on the side of the body. Lateral displays are typically performed in aggressive interactions and involve two fish orienting parallel to each other in close proximity and vigorously shaking their bodies. During lateral displays the jaw is distended, opercula are spread and all fins are erect. In all female–female exposure trials, aggressive behaviors of both the focal gravid female and the stimulus females (gravid or brooding) were quantified to better characterize the reciprocal interactions between focal and stimulus fish.

#### Tissue preparation

Focal females remained in the center stimulus compartment to interact with stimulus fish (or isolated during empty compartment

trials) for 30 min after the end of the 30 min stimulus period and were then anesthetized in ice-cold fish water and killed by rapid cervical transection. Brains were removed and fixed in 4% paraformaldehyde in 1× phosphate buffered saline (1× PBS) overnight, rinsed in 1× PBS and cryoprotected in 30% sucrose prior to sectioning. Brains were mounted in OCT media (TissueTek, Sakura, Torrance, CA, USA) and sectioned in the transverse plane at  $20 \mu\text{m}$  on a cryostat, collected onto alternate charged slides (Superfrost plus, VWR, Radnor, PA, USA), allowed to adhere for two nights at room temperature and then stored at  $-80^\circ\text{C}$  until staining.

#### *cfos* in situ hybridization

To test the hypothesis that brain activation patterns of gravid females differ based on social context, we used chromogenic *in situ* hybridization with riboprobes designed to label the IEG *cfos* mRNA as described previously (Butler and Maruska, 2016). Briefly, commercially synthesized *A. burtoni cfos* primers were used to generate a probe template by PCR amplification, and a transcription reaction was used to incorporate digoxigenin (DIG)-labeled nucleotides into the purified PCR template before probe purification. The probe was diluted 1:5 in hybridization buffer and stored at  $-20^\circ\text{C}$  until use. Probe specificity was previously demonstrated (Butler and Maruska, 2016).

Slides with brain sections were brought to room temperature, rinsed in 1× PBS ( $3 \times 5$  min), fixed for 10 min in 4%

paraformaldehyde (PFA) in 1× PBS, rinsed in 1× PBS (2×5 min), incubated in proteinase K solution for 10 min, rinsed with 1× PBS for 10 min, fixed with 4% PFA for 20 min, rinsed again with 1× PBS (2×5 min) and then rinsed with Milli-Q water prior to incubation in 0.1 mol l<sup>-1</sup> triethanolamine-HCl (pH 8.0) with 0.25% acetic anhydride (1:400 final concentration) for 10 min. Slides were then rinsed with 1× PBS prior to incubation in pre-warmed hybridization buffer for 2–3 h in a sealed humidified chamber at 60–65°C. Tissue was then hybridized with DIG-labeled probe at 60–65°C in a sealed humidified chamber overnight (13–18 h). Sections were washed at 60–65°C in 2× sodium citrate chloride (SSC):50% formamide (2×30 mins), 1:1 mixture of 2× SSC:maleate buffer (MABT) (2×15 mins) and then MABT (2×10 min). Slides were then washed with MABT at room temperature (2×10 min), and non-specific binding was blocked with MABT containing 2% bovine serum albumin (BSA) for 3 h at room temperature. Slides were then incubated with anti-DIG antibody (Sigma-Aldrich, St Louis, MO, USA) at 1:5000 in blocking solution (MABT with 2% BSA) at 4°C overnight in a humidified chamber. Slides were rinsed with MABT (3×30 min) at room temperature, followed by washes with alkaline phosphatase (AP) buffer (2×5 min) and then developed in the dark at 37°C with nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate (Sigma-Aldrich) in AP buffer for 2–5 h. Following development, slides were rinsed with 1× PBS (3×5 min), fixed with 4% PFA for 10 min, rinsed with 1× PBS (3×5 min) and mounted with aqueous mounting media (Aqua-mount, Fisher Scientific, Waltham, MA, USA).

### Quantification of brain activation

To quantify differences in the number of *cfos*-expressing cells in the brain, slides were visualized on a Nikon Eclipse Ni microscope and photographs were taken with a color digital camera controlled by Nikon Elements software (Tokyo, Japan). Bright field and phase contrast were used to visualize neuroanatomical markers and brain nuclei in relation to DIG-labeled cells. Quantification was done by an observer that was blind to experimental condition. Final images were adjusted for levels, contrast and brightness as needed in Adobe Illustrator CC (v21.10; San Jose, CA, USA). A Cresyl Violet-stained *A. burtoni* reference brain, *A. burtoni* brain atlas and other relevant papers (Fernald and Shelton, 1985; Maruska et al., 2017; Munchrath and Hofmann, 2010) were used for the identification of neuroanatomical structures. Stereotactical and neuroanatomical markers were used to designate the borders and rostro-caudal extent of each region to ensure consistency across animals. The following regions of the SDMN were quantified: ventral nucleus of ventral telencephalon (Vv); rostral portion of the ventral nucleus of the telencephalon (rVv); supracommissural nucleus of the ventral telencephalon (Vs); dorsal part of the ventral telencephalon (Vd); anterior tuberal nucleus (ATn); anterior part of the periventricular preoptic nucleus (nPPa); parvocellular division of the magnocellular preoptic nucleus (nPMp); magnocellular division of the magnocellular preoptic nucleus (nMMp); and lateral nucleus of dorsal telencephalon, granular region (DI-g). Other regions of the SDMN [central part of the dorsal telencephalon (Dc), periventricular nucleus of the posterior tuberculum (TPp), medial portion of dorsal telencephalon (Dm), periaqueductal gray (PAG), ventral tuberal nucleus (VTn), central part of ventral telencephalon (Vc), and lateral part of the ventral telencephalon (VI)] were not quantified either because of their small size, inconsistent labeling or inability to reliably identify the region based on surrounding neuroanatomical markers. The posterior nucleus of the dorsal telencephalon (Dp) was also quantified because it is an important

olfactory processing region (Satou, 1990). Images were taken at the highest magnification (×10 or ×20 objective) that encompassed the entire area of interest. For ×10 images (Dp, Vs, DI-g), nuclei borders were outlined with 50×50 μm gridlines applied to each image. The number of *cfos*-expressing cells in five random boxes per section was quantified manually, and cell density was calculated by dividing the number of cells within the boxes by the area of the boxes. Criteria for quantification included identification of stained cells with a clear discernible border, visible nucleus and could easily be distinguished from adjacent cells. For ×20 images (rVv, Vv, Vd, ATn and preoptic nuclei), the same procedure was followed, except that outlined nuclei borders were overlaid with 15×15 μm gridlines, and the number of cells in three random boxes per section were quantified. Four consecutive sections were quantified for each region and averaged together for a cell density value (number of cells μm<sup>-2</sup>) of that region in a particular animal. These values were then averaged across individuals within each social exposure treatment (i.e. dominant male, brooding female, juvenile and empty compartment exposures). Gravid female exposure condition was not included in the brain activation analysis because it did not significantly differ from juvenile exposure in any brain region investigated (ANOVA, *P*>0.05). In addition, both urination and social behaviors of focal females did not differ between these groups (ANOVA, *P*>0.05) so both conditions were deemed of low social salience.

### Statistical analyses

To compare chemosensory signaling among focal gravid females exposed to different social contexts, urination rate data were square-root-transformed to pass normality and equal variance, followed by two-way repeated-measures ANOVA (factors: social context and pre-/post-stimulus environment) with Student–Newman–Keuls (SNK) *post hoc* tests. To compare differences in aggressive behavior across females, data were square-root-transformed to pass normality followed by one-way ANOVA with SNK *post hoc* tests. Data that could not be normalized by transformation were compared with non-parametric tests (Kruskal–Wallis one-way ANOVA on ranks). Significant outliers detected by Grubb's test were removed for data analysis (urination rate: one outlier removed from empty compartment condition, one outlier removed from brooding female condition). Outliers removed for urination rate were also subsequently removed from social behavior (aggressive displays) analysis. Pearson correlation tests were used to examine the relationship between urination rate and aggressive displays in focal gravid females exposed to different social stimuli. To examine differences in neural activation, the density of *cfos*-expressing cells in regions of the SDMN were analyzed by one-way ANOVA with SNK *post hoc* tests. Some activation data were square-root-transformed to pass normality, and data that could not be normalized by transformation were compared with non-parametric tests (Kruskal–Wallis one-way ANOVA on ranks). Pearson correlations were used to examine correlations between *cfos* staining of each brain nucleus against other brain regions, and comparison heat maps were generated for high (dominant male, brooding female) and low (juveniles, empty compartment) social salience conditions. Discriminant function analysis (DFA) was used to group animals based on brain activation patterns (*cfos*-expressing cell density), and missing values (statistical outliers) were replaced with the group mean. All groups were considered equal during the classification, and within-group covariance was used to determine classification. Statistical comparisons were made in SigmaPlot 12.0 (San Jose, CA, USA) and SPSS (Chicago, IL, USA).

## RESULTS

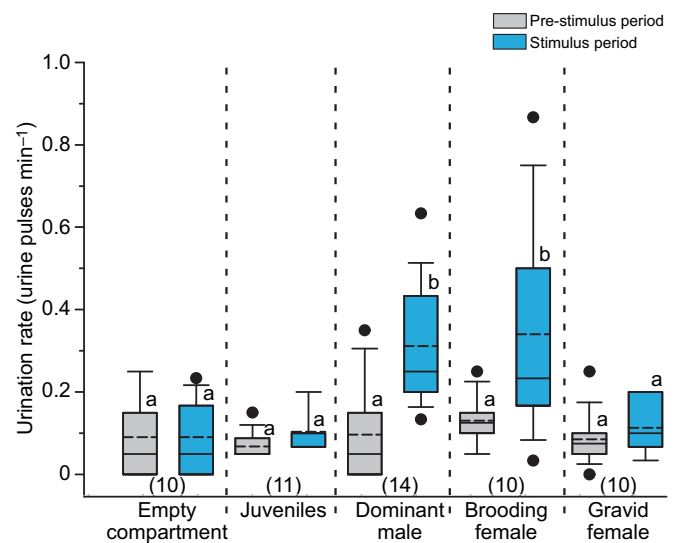
**Context-dependent chemosensory signaling and aggressive displays**

Gravid females showed context-dependent urine release, which was observed from all focal females as blue plumes released from the urogenital opening that were distinct from feces (Fig. 1C). Urine pulse duration ranged from short (<1 s) to long (up to 19 s). The volume of urine released also varied but was not quantified. Urine release by focal females did not simultaneously occur with aggressive behavior, occurring only at times of slow swimming or when the fish paused and hovered in the water column as observed in *A. burtoni* males (Maruska and Fernald, 2012) (Fig. 1D).

Urination behavior differed by social context (two-way repeated-measures ANOVA,  $F_{4,50}=4.774$ ,  $P=0.002$ ) and stimulus environment (pre-stimulus period/stimulus period) (two-way repeated-measures ANOVA,  $F_{1,50}=35.133$ ,  $P<0.001$ ), and there was a significant interaction between social condition and stimulus environment (two-way repeated-measures ANOVA,  $F_{4,50}=7.97$ ,  $P<0.001$ ). Across all 20 min pre-stimulus periods, urine release from focal females was not significantly different and ranged from 0 to 0.4 pulses  $\text{min}^{-1}$  ( $P>0.05$ ). Urination rate increased from the 20 min pre-stimulus period to the 30 min stimulus period when focal females were exposed to certain social conditions; when interacting with a dominant male or a brooding female ( $P<0.05$ ), and did not change when in a compartment alone, with another gravid female or with juveniles (all  $P>0.05$ ). Across all 30 min stimulus periods, focal gravid female urination rate was significantly higher during exposure to a dominant male or brooding female ( $P<0.05$ ) compared with when in a compartment alone, with another gravid female or with juvenile fish (all  $P>0.05$ ), and did not differ between exposure to a dominant male or brooding female ( $P>0.05$ ) (Fig. 2). Thus, gravid *A. burtoni* females alter their urination rates in both inter- and intra-sexual contexts.

The vast majority of aggressive displays (bites and lateral displays combined) by focal gravid females were directed towards other females (Fig. 3) (lateral displays were never conducted towards males, and only a few investigatory bites towards dominant males occurred in the first 1–2 min of the trial). Aggressive displays never occurred towards juveniles or in an empty compartment. Focal gravid females showed significantly more aggressive displays towards brooding females than towards gravid females (one-way ANOVA,  $F_{3,32}=9.67$ ,  $P<0.01$ , SNK  $P<0.05$ ) (Fig. 3A). The number of aggressive displays performed by the focal female ranged from 0 to 33 ( $19.0\pm 10.1$ ) toward stimulus gravid females and from 28 to 105 ( $78.5\pm 29.9$ ) towards stimulus brooding females. In 90% of trials with brooding females, the brooder initiated the aggression. In trials with brooding females, 100% of focal gravid females continued aggressive displays into the final 3 min of the trial, whereas only 27% (3 of 11 trials) of brooding females showed aggression lasting into the final 3 min of the trial. In interactions with other gravid females, the aggressive displays of focal gravid females ended prior to the last 10 min of the 30 min trial in 72% of trials (8 of 11 trials), whereas the stimulus gravid female aggression ended prior to the last 10 min of the trial in 55% of trials (6 of 11 trials) (see Fig. 3C as example of aggressive interactions).

The number of urine pulses released by focal gravid females was positively correlated with the total number of aggressive behaviors for exposure to brooding females and exposure to gravid females combined (Pearson correlation,  $r=0.89$ ,  $P<0.01$ ) and when separated by exposure group (Pearson correlations, gravid exposure:  $r=0.83$ ,  $P<0.01$ ; brooder exposure:  $r=0.89$ ,  $P<0.01$ ) (Fig. 3B).

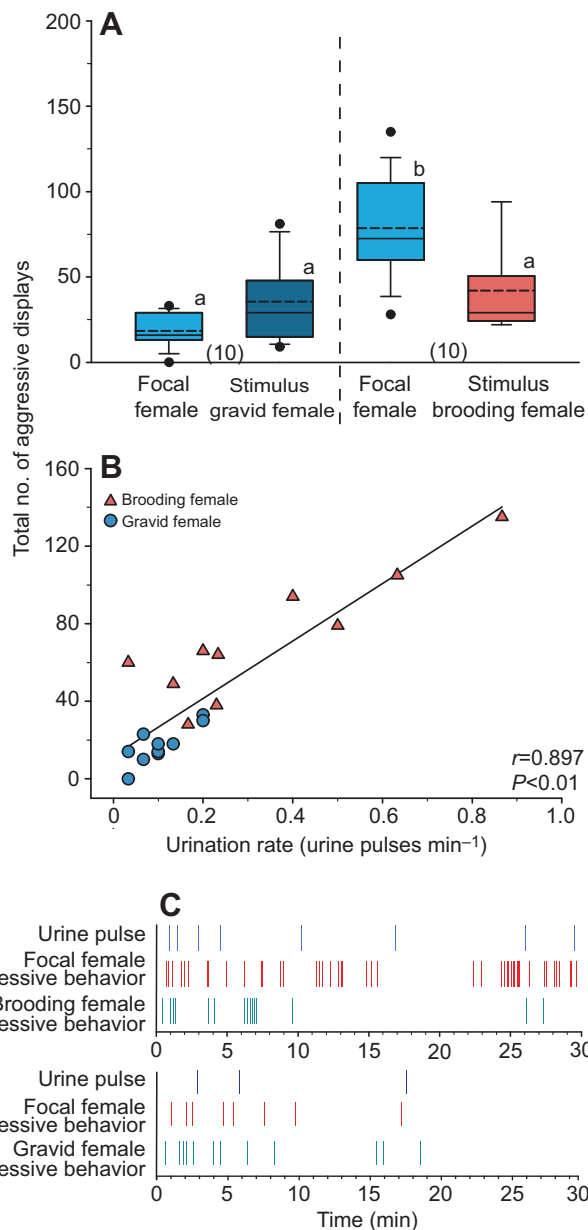


**Fig. 2. Gravid *A. burtoni* females show context-dependent chemosensory signaling.** Urination rate (number of urine pulses  $\text{min}^{-1}$ ) of gravid females did not differ across all 20 min pre-stimulus trials. Urination rates in focal gravid females increased from the 20 min pre-stimulus period when exposed to dominant males and brooding females during the 30 min stimulus period. There was no increase in urination rate from the pre-stimulus to stimulus period when focal females were exposed to other gravid females, juveniles or an empty compartment. Different letters indicate statistical significance at  $P<0.05$ . Box plots were used to represent data: data median is represented by solid line and data mean is represented by a dashed line. The box extends to the furthest data points within the 25th and 75th percentiles, and whiskers extend to the 10th and 90th percentiles. Absence of whiskers indicates absence of data points outside the 25th/75th percentiles. Closed circles represent values outside the 5th and 95th percentiles. Sample sizes (number of trials) are shown in parentheses.

**Brain activation patterns in the SDMN**

Focal gravid females showed context-specific neural activation, quantified as the density of *cfos*-expressing cells, in certain regions of the SDMN. For example, focal gravid females had significantly higher *cfos* cell densities in the most rostral portion of Vv (rVv) (one-way ANOVA,  $F_{3,20}=15.08$ ,  $P<0.01$ , SNK  $P<0.05$ ), in Vd (one-way ANOVA,  $F_{3,20}=28.07$ ,  $P<0.01$ , SNK  $P<0.05$ ), in nPPa (Kruskal–Wallis one-way ANOVA on ranks,  $H=15.739$ , d.f.=3,  $P<0.05$ ) and in Vs (Kruskal–Wallis one-way ANOVA on ranks,  $H=17.872$ , d.f.=3,  $P<0.01$ ) when exposed to dominant males (reproductive context) compared with other contexts (Fig. 4). Focal gravid females showed greater *cfos*-expressing cell densities in ATn when exposed to brooding females (aggressive context) compared with other contexts (one-way ANOVA,  $F_{3,20}=16.9$ ,  $P<0.01$ , SNK  $P<0.05$ ) (Fig. 5). Higher densities of *cfos*-expressing cells occurred in the DI-g after exposure to both reproductive and aggressive contexts (one-way ANOVA,  $F_{3,22}=8.127$ ,  $P<0.01$ , SNK  $P<0.05$ ) (Fig. 5). There were no differences in *cfos*-expressing cell densities across contexts in the caudal Vv, Dp, nPMp or nMMp.

To investigate functional connectivity in the SDMN (and Dp), we created heat maps using Pearson correlations of *cfos* staining in these brain regions (Fig. 6). We investigated connectivity in high socially salient conditions (exposure to dominant males and brooding females) (Fig. 6A) and low social salience (exposure to an empty compartment and juvenile fish) (Fig. 6B). Organization of the heat maps was based on hierarchical clustering of highly salient conditions and then also applied to low salience conditions. We found that activation among brain regions differed between these



**Fig. 3. Gravid female *A. burtoni* show more aggression towards brooding females, and aggression is positively correlated with urine release.**

(A) Focal gravid females show significantly more aggressive displays (bites and lateral displays) when exposed to a brooding female compared with when exposed to another gravid female. Different letters indicate statistical significance at  $P<0.05$  (one-way ANOVA). See Fig. 2 for box plot descriptions. (B) The urination rate of gravid females was positively correlated with the number of aggressive displays when exposed to both gravid and brooding females. (C) Example of temporal sequence exhibited by focal gravid females showing greater urination and aggressive displays when exposed to a brooding female (top) compared with another gravid female (bottom). Each vertical mark represents a urine pulse by the focal gravid female or an aggressive display (bites and lateral displays) by the focal or stimulus female during the 30 min stimulus period.

contexts. For example, rVv, Vv, Vs, Vd and nPPa were all positively correlated with each other during social contexts (with the exception of no significant correlation between nPPa and Vv) but not during low social salience contexts. Further, ATn was negatively correlated with these same regions (rVv, Vv, Vs, Vd, nPPa) during social contexts. In contrast, DI-g, Dp, nMMp and nPMp were not

correlated with the rest of the SDM during social contexts. In the low salience conditions, Vv or rVv was positively correlated with Vd, ATn, Dp and nMMp, whereas nMMp was negatively correlated with nPMp, DI-g and ATn. Table 1 shows Pearson correlation coefficients and  $P$ -values for females exposed to empty compartments and juveniles (high social salience). Table 2 shows Pearson correlation coefficients and  $P$ -values for females exposed to dominant males and brooding females (low social salience conditions). Focal female urination behavior did not correlate with neural activation in any brain region and thus was not included in the matrices. Aggressive behavior only occurred in specific social contexts (exposure to brooding or gravid females) and therefore was also not included in the matrix analysis.

In reproductive contexts (exposure to dominant male), the density of *cfos*-expressing cells in the rVv, Vd, Vs and nPPa was greater than in all other contexts. Greater *cfos*-expressing cell densities were also seen in the ATn during intra-sexual aggressive contexts and in the DI-g in both reproductive and aggressive contexts. Fig. 7A shows a sagittal view of the brain of *A. burtoni* to summarize regions of the SDM showing differential *cfos*-expression in focal gravid females exposed to different social contexts.

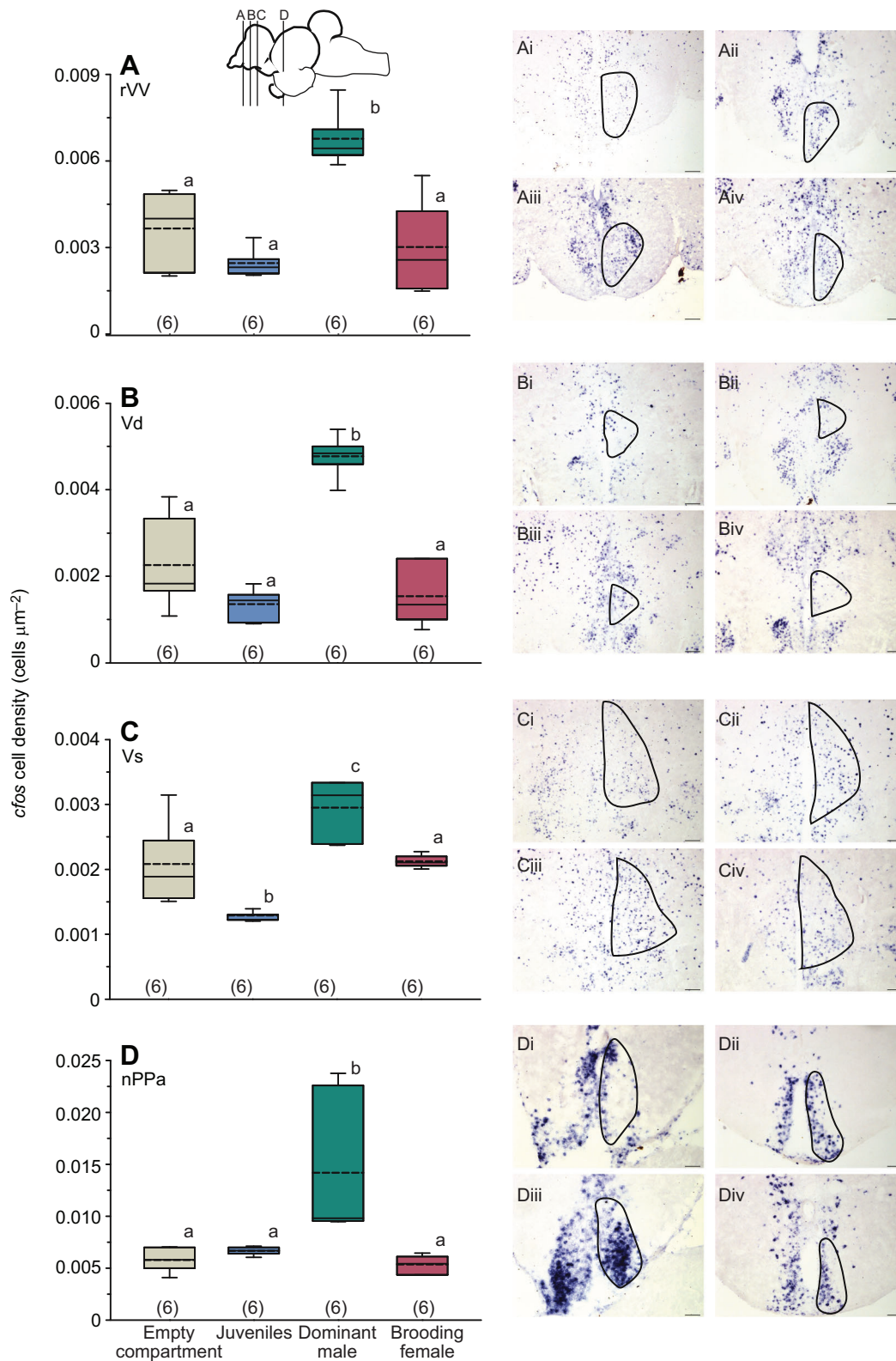
To further examine differences in activation of the SDM in different social contexts, we performed canonical DFA (Fig. 7B). DFA determines which variables (brain regions) may contribute to the sorting of animals into different groups and determines if animal group can be distinguished based on brain activation patterns alone. Our DFA correctly predicted 100% of animals in all four groups (exposure to empty compartment, juveniles, dominant males and brooding females). Functions 1 and 2 were significant ( $P<0.05$ ). Function 1 was driven by the rVv, Vd and Vs, and explained 65.8% of the variance. Function 2 was driven by the ATn, Vs and DI-g, and explained 30.4% of the variance. Functions 1 and 2 accounted for more than 96% of the variance and correctly classified and separated the four exposure groups based on brain activation alone.

## DISCUSSION

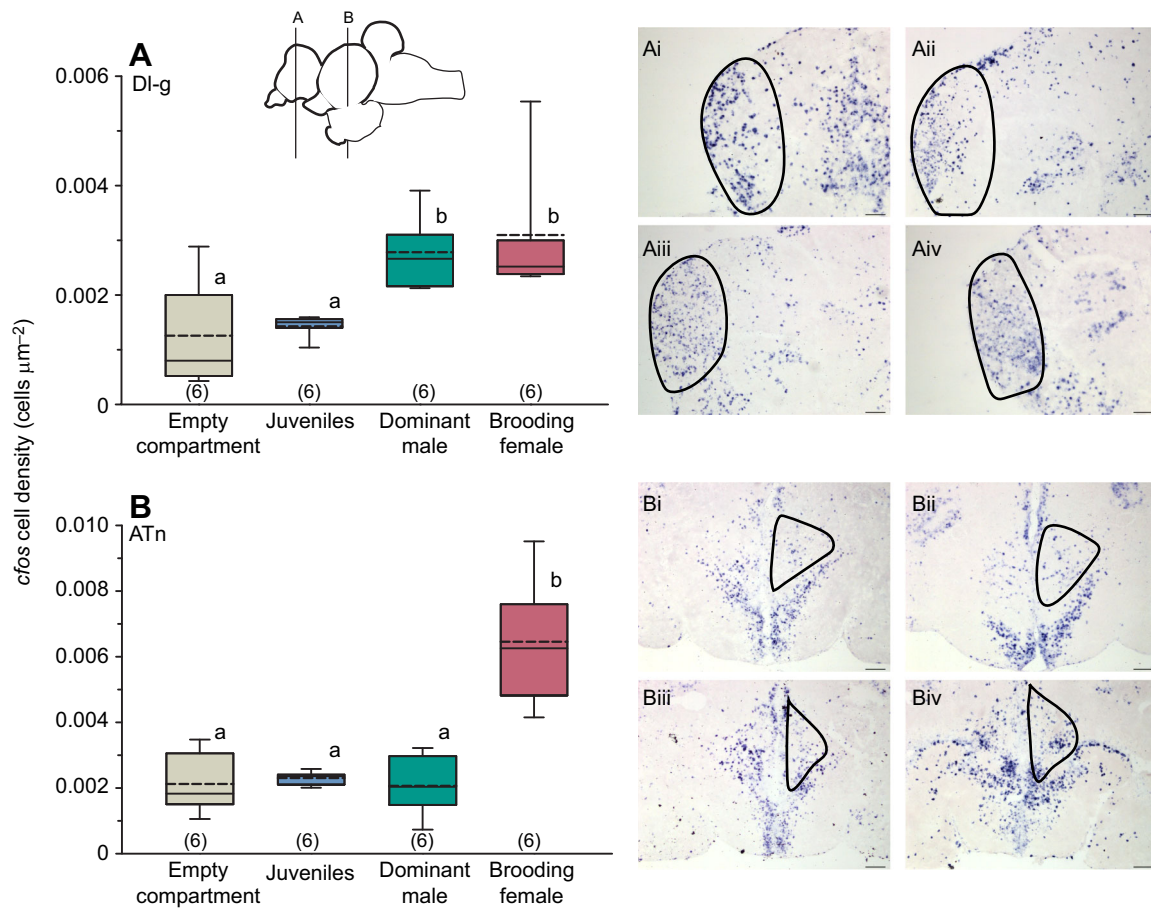
Here, we demonstrate that gravid female *A. burtoni* exhibit context-dependent chemosensory signaling, social behaviors and neural activation patterns during inter- and intra-specific social interactions. Gravid females increase their urination rate in aggressive and reproductive contexts, and increase aggression when exposed to brooding but not other gravid females. Using *in situ* hybridization for the IEG *cfos*, we identified differential neural activation in specific SDM nodes following exposure to various social environments, as well as altered functional connectivity of the SDM following high compared with low social salience.

### Context-dependent chemosensory signaling and behavior

Gravid *A. burtoni* females increase their urination in the presence of dominant males and brooding females, representing both inter- and intra-sexual contexts. Several fish species use chemical signals during reproduction and social interactions, conveying reproductive state and social status to conspecifics (Hara, 1994; Stacey, 2011). In fact, it is hypothesized that fishes were predisposed to developing a pheromone signaling system because gonadally produced metabolites indicative of reproductive state and/or social status are readily released into the water and detected by conspecifics (Doving, 1976). Freshwater fishes are hyperosmotic to their environment and must expel water via urination to maintain homeostasis; release of urinary pheromones therefore provides an opportunity to communicate important information to conspecifics (Stacey, 2011). The active control of pheromone release via urine occurs in several freshwater



**Fig. 4. Regions of the social decision-making network that show context-dependent *cfos* expression in gravid *A. burtoni* females during reproductive contexts.** (A–D) Gravid females show greater *cfos*-stained cell densities in rostral Vv (A), Vd (B), Vs (C) and nPPa (D) when exposed to dominant males (reproductive context). Photographs show representative examples of *cfos*-positive staining (purple dots) in each region after exposure to empty compartment (i), juvenile fish (ii), dominant males (iii) and brooding females (iv). Scale bars in A and B, 100  $\mu\text{m}$ ; scale bars in C and D, 50  $\mu\text{m}$ . The approximate quantified areas are outlined in each photograph. Different letters indicate statistical significance at  $P < 0.05$  (one-way ANOVA). See Fig. 2 for box plot descriptions. rVV, rostral portion of the ventral nucleus of ventral telencephalon; Vd, dorsal part of the ventral telencephalon; Vs, supracommissural nucleus of ventral telencephalon; nPPa, anterior part of the periventricular preoptic nucleus.



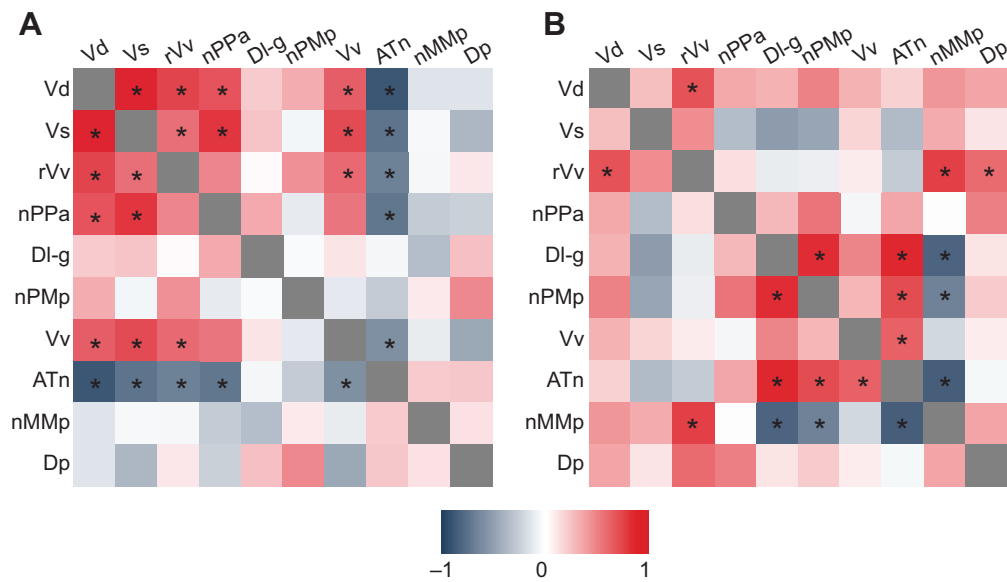
**Fig. 5. Regions of the social decision-making network that show context-dependent *cfos* expression in gravid *Astatotilapia burtoni* females during aggressive and/or reproductive contexts.** (A) A higher density of *cfos*-expressing cells was observed in DI-g in both reproductive and aggressive contexts compared with control conditions. (B) Greater *cfos* expression was seen in ATn in response to brooding females (aggressive context) compared with the reproductive context and control conditions. Photographs show representative example *cfos*-positive staining (purple dots) with outlines demonstrating the quantified area in each region after exposure to empty compartment (i), juvenile fish (ii), dominant males (iii) and brooding females (iv). Scale bars: 100  $\mu\text{m}$ . Different letters indicate statistical significance at  $P < 0.05$  (one-way ANOVA). See Fig. 2 for box plot descriptions. DI-g, lateral nucleus of dorsal telencephalon; ATn, anterior tuberal nucleus.

fish species as a means of signaling reproductive fitness, readiness and social dominance. Male swordtail fish (*Xiphophorus* spp.), for example, urinate more frequently in the presence and proximity of females (Rosenthal et al., 2011). Similarly, Mozambique (*Oreochromis mossambicus*) and Nile (*Oreochromis niloticus*) tilapia both use chemical signals during social interactions (Barata et al., 2007). Similar to these species, *A. burtoni* males alter their urination in social contexts, showing increased urination towards females (reproductive context) as well as towards rival males during aggressive interactions (Maruska and Fernald, 2012). Our results presented here demonstrate that gravid *A. burtoni* females also actively increase their urination during reproductive and aggressive interactions, providing evidence for the use of urine as a social signal in both sexes.

Female-released compounds of several fish species induce robust behavioral and physiological responses in male receivers (Stacey, 2011). The mechanism by which these relevant compounds are released by females varies considerably across species. For example, whereas female goldfish (*Carassius auratus*) will purposely alter when and where they release urine pulses during courtship, female Mozambique tilapia (*O. mossambicus*) do not alter their urination rate in the presence of males, suggesting they do not actively advertise their reproductive state (Almeida et al., 2005; Appelt and Sorensen,

2007). Mozambique tilapia exhibit a very similar reproductive strategy to that of *A. burtoni* with dominant males actively defending territories, and courting and spawning with females, followed by maternal mouth brooding. Thus, while *A. burtoni* females actively alter their urination rate, female Mozambique tilapia do not, suggesting that the use of urine as a social signal evolved differently in two closely related cichlid species. In fact, it was suggested that chemical signaling and the ability to detect species-specific chemical signals may serve as a driving force of speciation in cichlids of the rift valley lakes of Africa (Nikaido et al., 2014). While female-released chemical cues are investigated in reproductive contexts (Almeida et al., 2005; Appelt and Sorensen, 2007; Miranda et al., 2005), there is a paucity of information regarding chemosensory signaling by females in other social contexts, including aggression. The increased aggression and urination rate in *A. burtoni* during only one of the two intra-sexual interactions provides further evidence for the use of urine as a true social signal. Coupled with previous research in *A. burtoni* males, these data shed light on how chemosensory communication is used between both sexes of a single species, providing us with valuable information to generate more accurate models of how chemosensory signals might be integrated with other sensory modalities to elicit appropriate social behaviors and how these behaviors may have evolved.





**Fig. 6. Females exposed to high and low social salience conditions have different co-activation in brain regions involved in social behavior.** Heat maps of Pearson correlation coefficients ( $r$ =color scale) of *cfos* staining in brain nuclei in females exposed to (A) high social salience environments (dominant males and brooding females) and (B) low social salience environments (empty compartment and juvenile fish). Gravid females interacting in high social salience situations reveals a functional network within the SDMN involving rVv, Vv, Vs, Vd, nPPa and ATn, which is absent during low social salience. Sample size was 12 fish per brain region for both conditions. Asterisks indicate significance ( $P < 0.05$ ). SDMN, social decision-making network; Vv, ventral nucleus of ventral telencephalon; rVv, rostral portion of Vv; Vd, dorsal part of the ventral telencephalon; Vs, supracommissural nucleus of ventral telencephalon; nPPa, anterior part of the periventricular preoptic nucleus; ATn, anterior tuberal nucleus; nMMp, magnocellular division of the magnocellular preoptic nucleus; nPMp, parvocellular division of the magnocellular preoptic nucleus.

In addition to urination behavior, we observed context-dependent aggression in gravid females during intra-sexual interactions. Interestingly, this aggression was significantly higher towards brooding females compared with other gravid females. *Astatotilapia burtoni* females will engage in male-typical aggressive displays and even form dominance hierarchies in the absence of males, but the underlying causes of the brooder-centered aggression remain unclear (Renn et al., 2012). Female Nile tilapia exhibit increased

maternal aggression with the progression of their brood cycle (Oliveira and Almada, 1998). Similarly, in 90% of trials conducted in our study, the brooding female initiated the aggression. This may be due to protective maternal care behaviors by the brooding female, followed by reciprocated aggression from the gravid female. Another explanation could be what is termed ‘the Desperado effect’ where extreme consequences can lead to lower quality individuals showing heightened aggression (Grafen, 1987).

**Table 1. Pearson correlation coefficients of *cfos* staining in brain nuclei of focal gravid *Astatotilapia burtoni* females exposed to high social salience conditions**

		Vs	rVv	nPPa	DI-g	nPMp	Vv	ATn	nMMp	Dp
Vd	R	<b>0.862</b>	<b>0.743</b>	<b>0.68</b>	0.207	0.333	<b>0.636</b>	<b>-0.88</b>	-0.15	-0.15
	P	<b>&lt;0.01</b>	<b>0.01</b>	<b>0.01</b>	0.542	0.293	<b>0.02</b>	<b>&lt;0.01</b>	0.650	0.667
Vs	R		<b>0.573</b>	<b>0.796</b>	0.236	-0.06	<b>0.719</b>	<b>-0.73</b>	-0.05	-0.37
	P		<b>0.05</b>	<b>&lt;0.01</b>	0.484	0.849	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.888	0.259
rVv	R			0.479	0.032	0.444	<b>0.593</b>	<b>-0.67</b>	-0.05	0.1
	P			0.116	0.926	0.148	<b>0.04</b>	<b>0.02</b>	0.87	0.769
nPPa	R				0.343	-0.11	0.543	<b>-0.72</b>	-0.27	-0.24
	P				0.301	0.731	0.06	<b>&lt;0.01</b>	0.404	0.47
DI-g	R					-0.03	0.11	-0.06	-0.34	0.25
	P					0.93	0.748	0.864	0.925	0.49
nPMp	R						-0.12	-0.26	0.089	-0.06
	P						0.71	0.407	0.78	0.864
Vv	R							<b>-0.58</b>	-0.11	-0.45
	P							<b>0.05</b>	0.737	0.167
ATn	R								0.213	0.244
	P								0.505	0.508
nMMp	R									0.124
	P									0.716

Data represent correlation coefficients (R) and  $P$ -values (P) from focal gravid females exposed to the dominant male and brooding female conditions ( $N=12$  animals total) and were used to create the heat map in Fig. 6A. Bold indicates  $P < 0.05$ . Vv, ventral nucleus of ventral telencephalon; rVv, rostral portion of Vv; Vs, supracommissural nucleus of ventral telencephalon; Vd, dorsal part of the ventral telencephalon; ATn, anterior tuberal nucleus; nPPa, anterior part of the periventricular preoptic nucleus; nPMp, parvocellular division of the magnocellular preoptic nucleus; nMMp, magnocellular division of the magnocellular preoptic nucleus; DI-g, lateral nucleus of dorsal telencephalon, granular region; Dp, posterior nucleus of the dorsal telencephalon.

**Table 2. Pearson correlation coefficients of *cfos* staining in brain nuclei of focal gravid *A. burtoni* females exposed to low social salience conditions**

		Vs	rVv	nPPa	DI-g	nPMp	Vv	ATn	nMMp	Dp
Vd	R	0.26	<b>0.68</b>	0.35	0.31	0.49	0.31	0.18	0.42	0.361
	P	0.802	<b>0.01</b>	0.271	0.33	0.10	0.34	0.57	0.2	0.248
Vs	R		0.45	−0.34	−0.51	−0.47	0.17	−0.35	0.33	0.11
	P		0.163	0.30	0.10	0.149	0.62	0.289	0.32	0.75
rVv	R			0.13	−0.11	−0.10	0.09	−0.27	<b>0.75</b>	<b>0.58</b>
	P			0.68	0.74	0.77	0.79	0.404	<b>&lt;0.01</b>	<b>0.05</b>
nPPa	R				0.28	0.54	−0.06	0.35	0.02	0.51
	P				0.38	0.07	0.855	0.26	0.96	0.09
DI-g	R					<b>0.82</b>	0.48	<b>0.84</b>	<b>−0.81</b>	0.11
	P					<b>&lt;0.01</b>	0.115	<b>&lt;0.01</b>	<b>&lt;0.01</b>	0.73
nPMp	R						0.29	<b>0.71</b>	<b>−0.66</b>	0.21
	P						0.36	<b>0.01</b>	<b>0.02</b>	0.51
Vv	R							<b>0.63</b>	−0.21	0.08
	P							<b>0.03</b>	0.542	0.80
ATn	R								<b>−0.84</b>	−0.05
	P								<b>&lt;0.01</b>	0.881
nMMp	R									0.36
	P									0.27

Data represent correlation coefficients (R) and *P*-values (P) from focal gravid females exposed to juveniles and empty compartment conditions (*N*=12 animals total) and were used to create the heat map in Fig. 6B. Bold indicates *P*<0.05. Vv, ventral nucleus of ventral telencephalon; rVv, rostral portion of Vv; Vs, supracommissural nucleus of ventral telencephalon; Vd, dorsal part of the ventral telencephalon; ATn, anterior tuberal nucleus; nPPa, anterior part of the periventricular preoptic nucleus; nPMp, parvocellular division of the magnocellular preoptic nucleus; nMMp, magnocellular division of the magnocellular preoptic nucleus; DI-g, lateral nucleus of dorsal telencephalon, granular region; Dp, posterior nucleus of the dorsal telencephalon.

*Astatotilapia burtoni* females undergo a period of forced starvation during mouth brooding, which results in changes in fitness, behavior and physiology, resulting in lowered body condition (Grone et al., 2012; Oppenheimer, 1970). Thus, the heightened aggression observed in brooding females could be a result of the Desperado effect, with gravid females in turn responding with increased aggression. However, the brooding females ceased aggression before the end of the trial while the aggressive displays of the gravid females persisted throughout the entire 30 min trial in most instances, despite receiving no more aggression from the brooding female. Thus, while the initial aggression of the gravid female is likely to be a response to the brooding female's behavior, the persistence of this aggression, even when the brooder stops engaging in aggressive displays, suggests that it is not solely a responsive behavior. Further, in community environments, adult *A. burtoni* will often cannibalize newly released fry, so it is also possible that the developing fry in the mouth indicate a potential food presence, leading the gravid female to attack the brooding female in an attempt to eat the fry (K.E.F. and K.P.M., personal observation in small, experimental compartments only).

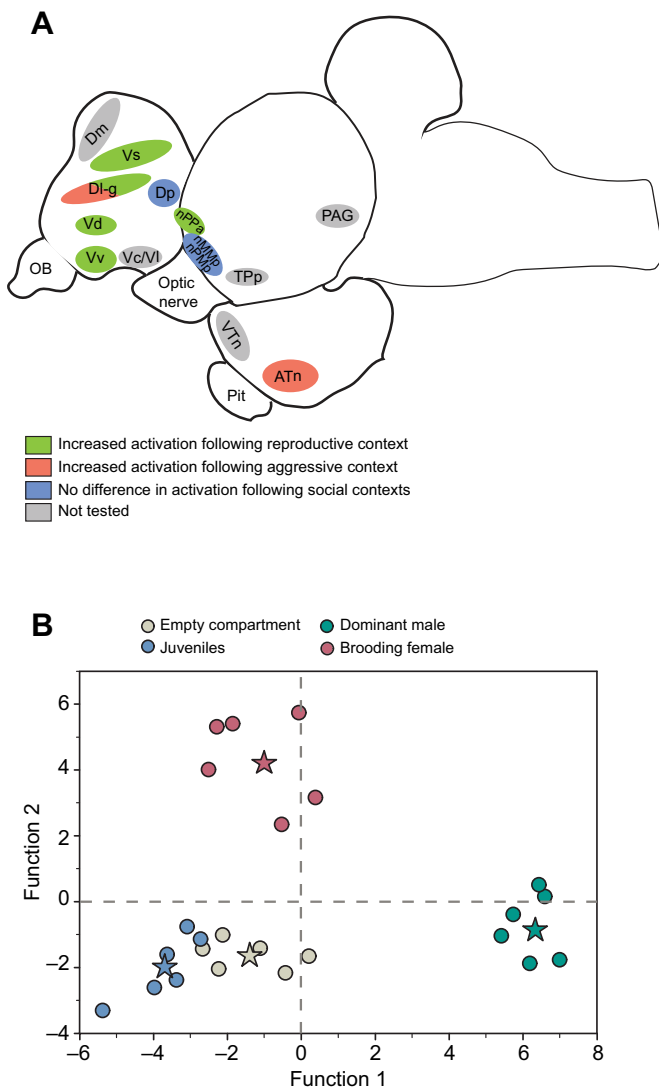
Aggressive displays between focal and stimulus *A. burtoni* gravid females typically occurred only during the first few minutes of the trial and were more investigatory in nature. The lack of aggression seen in these intra-sexual interactions may be due to the absence of a dominant male. The idea of divisive asymmetry states that low levels of resource availability may alter female–female contest rules (Grafen, 1987). In general, female–female competition is less understood than male–male interactions, but this phenomenon is observed in females across taxa. For example, in female paper wasps, when the value of resources is high, females will engage in escalated fighting behavior but not when resource value is low (Tibbetts and Lindsay, 2008). In our study, the absence of a male in the stimulus compartment may signal to gravid females that the cost of fighting one another may be too great without a receptive male to spawn with after winning a fight. While directed aggression between females is rarely observed in large communities of *A. burtoni* (K.E.F. and K.P.M., personal observations), the reduced

aggressive displays compared with gravid–brooding interactions may be a result of altered resource availability.

#### Context-dependent neural activation

In gravid female *A. burtoni*, we found context-dependent neural activation within specific SDMNs. All animals must process stimuli from the surrounding environment to make appropriate behavioral decisions; the SDMNs is hypothesized to integrate relevant social information that leads to adaptive behavioral responses (Goodson, 2005; O'Connell and Hofmann, 2011). Thus, contexts that differ in social valence should reveal differential activation in some SDMNs nodes. In fact, we found greater neural activation in the rostral portion of Vv, Vd, Vs and nPPa of gravid females exposed to a reproductive context (dominant male) compared with aggressive intra-sexual and control conditions. Further, in intra-sexual aggressive contexts, we found greater neural activation in ATn of focal gravid females and greater activation in DI-g following both reproductive and aggressive interactions. Thus, these data demonstrate some nodes of the SDMNs are individually involved in processing socially relevant information to elicit context-specific social behaviors.

In reproductive contexts, gravid *A. burtoni* females showed higher neural activation in several SDMNs regions, suggesting roles in processing sexually relevant social information. Importantly, two regions where greater neural activation was observed in reproductive contexts, i.e. Vv and Vs, are shared between the social behavior network and mesolimbic reward system that make up the SDMNs (O'Connell and Hofmann, 2011). Vv and Vs are putative homologs to the mammalian lateral septum and extended medial amygdala, respectively (Wullimann and Mueller, 2004). Fishes with lesions in these regions show reduced courtship and reproductive behaviors (Kyle and Peter, 1982; Satou, 1990). In *A. burtoni*, these regions expressed higher levels of estrogen and androgen receptor mRNA in males given the chance to rise in social rank, suggesting involvement in preparing for behaviors associated with dominance (Maruska et al., 2013). Vs is involved in processing the salience of sensory information to produce behaviors (Gray, 1999), and lesions



**Fig. 7. Discriminant function analysis and summary of brain activation patterns in regions of the social decision-making network (SDMN) of gravid *A. burtoni* females after exposure to different social contexts.**

(A) Schematic sagittal diagram of the brain showing regions of the SDMN with greater activation quantified by *cfos* expression following exposure to different social contexts. Regions in green (Vs, Vd, rostral Vv, DI-g and nPPa) showed greater activation following reproductive contexts, and regions in red (ATn and DI-g) showed greater activation following aggressive contexts. SDMN regions that did not differ with social context (blue) and those that were not quantified (gray) are also shown. Vv, ventral nucleus of ventral telencephalon; Vs, supra commissural nucleus of ventral telencephalon; Vd, dorsal part of the ventral telencephalon; ATn, anterior tubular nucleus; nPPa, anterior part of the periventricular preoptic nucleus; nPMp, parvocellular division of the magnocellular preoptic nucleus; nMMp, magnocellular division of the magnocellular preoptic nucleus; DI-g, lateral nucleus of dorsal telencephalon, granular region; TPP, periventricular nucleus of the posterior tuberculum; Dm, medial portion of dorsal telencephalon; PAG, periaqueductal gray; VTn, ventral tubular nucleus; Vc, central part of ventral telencephalon; VI, lateral part of the ventral telencephalon; Dp, posterior nucleus of the dorsal telencephalon; OB, olfactory bulb; Pit, pituitary. (B) Discriminant function analysis correctly classified all animals into their respective social exposure groups based solely on brain activation patterns, and clearly distinguished focal gravid females exposed to a reproductive context (along Function 1) and an aggressive context (along Function 2) from all other conditions. Stars represent group centroids of focal gravid females exposed to control empty compartment (gray), juveniles (blue), dominant males (green) and brooding females (red).

of Vs in goldfish result in impaired processing of sexually relevant olfactory signals (Kyle et al., 1982). Gravid *A. burtoni* females receive visual, chemical, mechanosensory and acoustic cues from courting males (Fernald, 1977; Maruska and Fernald, 2012; Maruska et al., 2012), highlighting the need to integrate multimodal information. Vs showed the highest *cfos* expression following exposure to dominant males, with no difference in expression after exposure to brooding females and an empty compartment, and the lowest expression was observed upon exposure to juveniles. This may be explained by the higher variation in *cfos* expression observed following exposure to an empty compartment (see Fig. 4), which may be due in part to a stress response. Greater activation in Vs of female *A. burtoni* after reproductive interactions may result from sensory stimulation received from dominant males when females are receptive and choosing mates.

The most rostral portion of Vv also showed greater activation following a reproductive interaction. In another study, *A. burtoni* females that watched a chosen mate lose a fight had higher *cfos* expression in Vv, suggesting a role in processing social information in anxiety-like contexts (Desjardins et al., 2010). Importantly, that study did not involve females engaging in any particular social behavior but only observing the chosen male engaging in contests with other males. Thus, those females only received social information as opposed to engaging in social interactions. This may suggest a different role for Vv in observing versus actually engaging in social interactions. Interestingly, in our study, the greater *cfos* expression in Vv was limited to the most rostral portion. When including the most caudal portion of Vv, there was no difference across social contexts, suggesting a subpopulation of cells in Vv may be involved in regulating social behaviors specifically during reproductive contexts. The rVv was also the most heavily loaded value for Function 1 in the DFA, confirming its role in social behavior in gravid *A. burtoni* females. Our data further support a role for Vv and Vs in integrating internal state with the social environment to elicit behavioral responses during reproductive situations because they are the two regions shared by the social behavior network and mesolimbic reward pathway.

Vd also showed greater *cfos* expression in gravid females exposed to the reproductive context of dominant males compared with other conditions. While the exact homolog of Vd is unknown, it was suggested that in teleosts it may function similarly (or perhaps in combination) to both the nucleus accumbens and striatal formation (O'Connell and Hofmann, 2011). The nucleus accumbens in mammals is involved in motivation and reward circuits, sexual reward and processing of sexually relevant chemosensory stimuli (Becker et al., 2001; Hosokawa and Chiba, 2005; Portillo and Paredes, 2004). In mammals, females exposed to sexually relevant chemosensory stimuli or sexual behavior show an increase in Fos protein expression in nucleus accumbens (Hosokawa and Chiba, 2007; Kollack and Newman, 1992; Robertson et al., 1991). It should be noted, however, that this homology has been disputed (Goodson and Kingsbury, 2013). Vd is also described as a homolog to the striatal formation in mammals, a region associated with goal-oriented action (O'Connell and Hofmann, 2011; Wullimann and Mueller, 2004). Female gray tree frogs with lesions in striatal formation are prevented from finding calling males, implying involvement in mating call responses (Walkowiak et al., 1999). As *A. burtoni* males use acoustic signals during courtship displays (Maruska et al., 2012), the greater activation in the female Vd when exposed to a dominant male could reflect the processing of acoustic information. Further, while the precise function of Vd in teleosts remains unclear, an increase in *cfos* expression in this region in

gravid females following reproductive interactions where males are releasing urine and sending acoustic signals provides further support for a role of Vd in processing rewarding sexually relevant chemosensory and/or auditory signals in *A. burtoni*.

Gravid *A. burtoni* females also showed an increase in the density of *cfos*-expressing cells after exposure to reproductive contexts in a nucleus of the preoptic area (POA), the parvocellular region (nPPa). We analyzed *cfos* expression in the nPPa as well as the parvocellular (nPMp) and magnocellular (nMMp) divisions of the magnocellular preoptic nucleus. The nPPa is a putative homolog of the paraventricular region of the preoptic area in mammals, while nMMp and nPMp may be homologous to the supraoptic region of the POA (Moore and Lowry, 1998). The POA has a well-established role in reproduction across vertebrates (O'Connell and Hofmann, 2011). Stimulation of the POA increases courtship and spawning behavior in both sexes of several fish species, while lesions reduce these behaviors (Demski and Knigge, 1971; Macey et al., 1974; Satou et al., 1984). In addition, the nPPa of *A. burtoni* contains both GnRH1 neurons that regulate the reproductive axis and arginine vasotocin (homolog of arginine vasopressin in mammals) cells that are involved in an array of social behaviors in fishes (Davis and Fernald, 1990; Goodson and Bass, 2001; Greenwood et al., 2008; Huffman et al., 2012). In *A. burtoni*, several previous studies examined activation of the entire POA following social encounters but the differential activation of specific preoptic nuclei was not investigated. For example, in *A. burtoni* males, greater activation of the POA was observed following fighting compared with courting (Loveland and Fernald, 2017), as well as in males given the opportunity to rise in social rank (Maruska and Fernald, 2010). In *A. burtoni* females, IEG expression increased in the POA after seeing their chosen male win a fight (Desjardins et al., 2010). In *A. burtoni* males, no effect of social stimulus on cFos was observed in the POA when males were socially stimulated in either a reproductive or aggressive context (O'Connell et al., 2013), highlighting a potential inter-sexual difference in POA function. However, these males were not engaged in full contact with stimulus fish as in our study and were therefore not receiving all sensory signals. The POA of female birds and amphibians also exhibits greater neural activation expression in social contexts (Bharati and Goodson, 2006; Bolhuis et al., 2001). In the present study, we found greater neural activation specifically in the nPPa, but not other POA regions, in gravid females following reproductive, but not aggressive, contexts. Our results in nPPa suggest a role for this POA sub-region in regulating reproductive interactions in reproductively receptive females.

Following aggressive interactions, gravid *A. burtoni* females had greater neural activation in ATn, a putative homolog to the mammalian ventromedial hypothalamus (VMH) (Forlano et al., 2005; Goodson, 2005). In the mammalian VMH, *cfos* expression also increases after aggressive interactions (Kollack-Walker and Newman, 1995; Lin et al., 2011). The ATn is reciprocally connected to the POA in fishes, suggesting an important role in social behavior but functional roles for ATn in teleosts remain unknown. In *A. burtoni*, the ATn is likely to be involved in the transition between male social states (Maruska et al., 2013), and in processing male–male aggressive-context mechanosensory signals detected by the lateral line system (Butler and Maruska, 2016). In our study, DFA shows that ATn strongly contributes to separating focal gravid females exposed to brooding females (with highest intra-sexual aggression) from all other groups along Function 1. Thus, ATn activation after aggressive encounters in gravid *A. burtoni* females further supports its role in processing socially relevant mechanosensory information and in mediating or responding to aggressive behaviors.

One region of the SDMN, DI-g, showed greater neural activation following both reproductive and aggressive interactions. DI is the putative homolog of the mammalian hippocampus and has known functions in spatial learning and memory (Folgueira et al., 2004; Rodríguez et al., 2002). The increase in activation in this region following high social salience contexts (both intra- and inter-sexual conditions) may reflect some level of social memory. The DI-g was also recently implicated in processing sensory input from stimulation of the mechanosensory lateral line system in *A. burtoni* (Butler and Maruska, 2016). Reproductive quiver displays by males and aggressive lateral displays by females both generate water movements that likely stimulate the lateral line system. Thus, greater activation in DI-g following interactions associated with more mechanosensory inputs further supports its role in processing socially relevant lateral line information.

IEG expression as a means to examine neural activation following acute behavioral interactions is well established in mammals and teleost fishes (Healy et al., 2013; Kovács, 2008). While functional connectivity of the SDMN better explains stable behavioral states, information on individual nodes can provide insight into the neural mechanisms regulating social behaviors (Teles et al., 2015). In addition, our heat maps from Pearson correlation coefficients demonstrate that females exposed to high social salience environments (reproductive and aggressive) have different functional connectivity compared with females in low social salience environments. rVv, Vv, Vs, Vd and nPPa were all positively correlated with each other during high social salience contexts (with the exception of no significant correlation between nPPa and Vv) but these correlations were not observed in low salience conditions, demonstrating that the functional connectivity of these regions changes with social valence. rVv, Vs, Vd and nPPa all showed greater activation following reproductive and/or aggressive contexts, and ATn was negatively correlated with these same regions during high salience contexts, despite greater activation following exposure to brooding females only. Further, DFA shows ATn is highly involved in aggressive contexts. While an exact function of the ATn in teleosts is unknown, our results show similarities to the mammalian VMH with ATn involvement in aggression, as well as processing social information in combination with those regions activated in reproductive contexts only (rVv, Vd, Vs, nPPa).

The DI-g, which had greater activation following high social salience conditions (reproductive and aggressive contexts), did not correlate with any other brain regions in high salient contexts. However, DI-g did positively correlate with ATn (as well as nMMp and nPMp) during low salience conditions. The greater DI-g activation could simply be a result of enhanced lateral line stimulation during social interactions in a small experimental tank (Butler and Maruska, 2016). However, the lack of correlation with any other brain region may also indicate that DI-g is not part of the functional network in females during high social salience environments. However, it should be noted that relevant brain regions of the SDMN that may better explain these results may not have been included in the current study. Urination rate did not correlate with *cfos* expression in any brain region, demonstrating that the neural activity observed here is likely to be a result of overall social environment rather than mediating specific behaviors like chemosensory signaling.

## Conclusions

In the current study, we show that gravid *A. burtoni* females use urine as a social signal by actively altering release in different social

contexts, and exhibit context-dependent aggression during intra-sexual interactions. While it was known that *A. burtoni* males contextually release urine, here, we report that females also utilize urine as a chemical messenger in a similar manner. Investigating chemical signaling in both sexes of a single species is imperative for a comparative understanding of chemical signaling across vertebrates. Our study shows that females are not only signaling receptivity to males (as seen in other fish species) but also signaling to other females during aggressive interactions, possibly to convey fitness. Further, while male aggression is relatively well studied in *A. burtoni*, female intra-sexual aggression is poorly understood. Here, we report increased aggression between females of different reproductive states compared with females of the same reproductive state. Examining neural activation patterns in gravid females exposed to reproductive and aggressive contexts, we identified several SDMN nodes that showed differential activation as well as changes in the functional connectivity of the SDMN following high versus low social salience conditions, providing further understanding of the SDMN's role in various social interactions. Our DFA of brain activation data correctly classified 100% of females by social exposure (empty compartment, juvenile, dominant male and brooding female), and the regions primarily driving this distinction were the rVv (reproductive) and ATn (aggressive). Coupled with previous reports, our data provide insights into how chemical signals may have helped shape the evolution of social communication in fishes, and sheds light on the neural correlates mediating context-specific inter- and intra-sexual social behaviors in a single fish species.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: K.E.F., K.P.M.; Methodology: K.E.F.; Validation: K.E.F., K.P.M.; Formal analysis: K.E.F.; Investigation: K.E.F.; Resources: K.P.M.; Data curation: K.E.F.; Writing - original draft: K.E.F.; Writing - review & editing: K.E.F., K.P.M.; Visualization: K.E.F., K.P.M.; Supervision: K.P.M.; Project administration: K.P.M.; Funding acquisition: K.E.F., K.P.M.

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