4-2016

Chemosensory Signals from Gravid Females Elicit Behavioral Response from Dominant Male Astatotilapia burtoni

Cristopher T. McVicker

Chemosensory Signals from Gravid Females Elicit Behavioral Response from Dominant Male *Astatotilapia burtoni*

by

Christopher T. McVicker

Undergraduate honors thesis under the direction of

Dr. Karen P. Maruska

Department of Biological Sciences

Submitted to the LSU Roger Hadfield Ogden Honors College in partial fulfillment of the Upper Division Honors Program.

April 2016

Louisiana State University
& Agricultural and Mechanical College
Baton Rouge, Louisiana

Introduction

Chemosignals, *i.e.*, chemical messages that are released by a sender and detected by a receiver, are used across animal taxa to convey social information such as reproductive receptiveness, social status, and sex to conspecifics, and may elicit behavioral and/or physiological responses [1-7]. Chemosensory signals are often integrated with other types of social signals, such as visual and acoustic cues, to create more complete profiles of conspecifics in the signal receiver's society, such that receiver organisms can use the multisensory information to respond appropriately in various social environments [2].

Much work has been done on the use of multiple or all of these signal types to convey social information in teleost fishes, the most numerous and diverse group of vertebrates [1, 2, 8, 5, 9, 10]. However, the way in which these chemosensory cues are processed in the brain is poorly understood, often referred to as part of the "dark matter" of social neurobiology [11]. Understanding the social role of chemosensory cues in teleosts and where in the brain they are processed is crucial to understanding the evolution of chemical signaling and its role in social behavior.

The chemosensory system comprises two branches: olfaction (smell) and gustation (taste). In most teleosts, olfaction is characterized by the use of olfactory epithelia to detect odorants from the external environment and transmit signals to the olfactory bulb; olfaction is known to be associated with an array of behaviors, including feeding and social interaction (aggressive and reproductive) [12]. Conversely, gustation, the other chemosense, utilizes gustatory cells in the mouth and along the body's length; traditionally, it has been associated mainly with feeding, but not reproduction [12]. Chemosignaling in teleosts is perhaps best understood in the goldfish *Carrasius auratus*, which utilizes pheromones as intersex messengers to initiate reproductive receptiveness and coordinate spawning [3], although many other organisms from a wide variety of species encompassing other fish to mammals utilize chemosensory signaling as well, for various purposes, including reproduction [1, 4, 5, 11].

Here, we used the African cichlid, *Astatotilapia burtoni* (Günther 1894), formerly *Haplochromis burtoni*, as our model organism; it is an excellent subject for examining chemical signals in behavior, as cichlids are highly social organisms that exhibit a wide variety of social behaviors, reproduction strategies, and methods of communication [2, 5-10, 13]. Furthermore, they exhibit the same territorial and reproductive behaviors whether they are members of a wild population in a natural setting or bred and observed within a laboratory [13]. *A. burtoni* are endemic to Africa's Lake Tanganyika, where their hierarchical society is composed of dominant males, subordinate males, and females. Males can and do reversibly switch between the dominant and subordinate phenotypes depending on the social environment [14, 15]. Dominant males compose ~10-30% of the population and have bright coloration as well as aggressive behavior exhibited in territory defense and courtship in a lek-like system in order to entice females to enter their territory to spawn [2, 13, 14]. Subordinate males resemble females in color and are reproductively suppressed (without losing the ability to spawn) by the dominants to be non-reproductive and non-territorial, instead focusing on survival and improved chances for future dominance potential [13, 14]. Females of this species are mouthbrooders, holding fertilized eggs in their mouths until development (~2 weeks) has completed, whence the fry are released [2, 13]. Females become gravid at around 13-14 weeks in age, at which time they are reproductively receptive to males and respond to courtship behavior [16].

Dominant male *A. burtoni* alter urination rate depending on social context, suggesting that they use urinary messages to convey dominance status towards competing males and reproductive condition to attractive females (*i.e.*, females that may be inclined to reproduce) [2]. A similar mechanism has been observed in gravid females of this species, which also alter their urination contextually, increasing urine pulses in the presence of males and brooding females compared to other gravid females, juveniles, or when alone [Field and Maruska, in prep]. Males can discern gravidity using the information conveyed in chemosignals from females, as dominant males (who were also given visual stimulation) exhibited reproductive behaviors (in attempt to court blind young *Tilapia* sp.) after administration of chemosensory cues from gravid females, but not when exposed to cues from non-receptive females [6]. Furthermore, it has also been shown that both sexes of *A. burtoni* possess olfactory systems that are responsive to conjugated

steroids, including some pregnanes that have been sulfated or glucuronidated, characteristics of sex pheromones [17, 18]. The contextual nature of chemosensory signal release, behavioral response of males to chemosensory cues, and olfactory sensitivity to pheromonal characteristics like sulfation, which is associated across vertebrate taxa with reproductive chemosensory communication [3, 17-20], suggest that gravid females release chemosensory cues that convey reproductive readiness to dominant males. How these chemosignals influence dominant male behavior and where they are processed in the brain, however, remain uninvestigated.

If dominant males register the reproductive readiness of females, they should respond with the appropriate social behaviors to initiate spawning. In the brain, the social behavior network (SBN) is a group of nuclei associated with a wide host of social behavior categories, including aggression, communication, offspring care, and reproduction [21]. The fact that the SBN brain nuclei all possess receptors for sex steroids implies that the SBN is capable of integrating social behavior exhibited by the organism with its own internal hormonal state to produce sexually differentiated and temporally appropriate behavior [21]. Furthermore, the SBN has been described across vertebrate taxa, allowing for consideration of neural processing of social behavior to occur from an evolutionary perspective via comparison of SBN function among various classes, which arose at different points [21]. In this way, examination of the social behavior network can provide insight into both how social behavior is processed within a single species as well as how and when such processing arose and was shaped by evolution.

In teleosts, many SBN nuclei are located in the telencephalon, an area associated with, among other things, male reproductive behaviors [21]. Furthermore, the telencephalon is located posteriorly adjacent to and receives neural input from the main processing center of olfaction, the olfactory bulb [21, 22]. In terms of specific nuclei, the ventral nucleus of the ventral telencephalon (Vv) is associated with reproductive behavior [21] and receives secondary afferent projections from the olfactory bulb [22], suggesting a role in mediating the reception of reproductive chemosensory cues sent by the opposite sex to produce responsive behavior in effort to court and spawn. The supracomissural nucleus of the ventral telencephalon (Vs), composed of both lateral (Vs-l) and medial (Vs-m) parts, like the Vv, is also innervated via the olfactory bulb,

although to a lesser extent [22], and associated with reproductive behavior as part of the SBN. The preoptic area is noted as an important area for a variety of social behaviors, with different nuclei responsible for different types; the anterior part of the parvocellular preoptic nucleus (nPPa), is one associated with reproduction [21], and it also receives innervation from the olfactory bulb [22]. Finally, the posterior nucleus of the dorsal telencephalon (Dp) is known to have the highest (of these regions) degree of innervation via secondary afferent neurons from the olfactory bulb and serves mainly to process olfactory signals after they travel through the olfactory bulb [22]. Thus, these regions of the SBN are prime candidates to examine for context-dependent activation from chemosensory signals.

Although some studies have shown the importance of acoustic [9] and the well-known visual [8, 10] signaling to cichlid reproduction, less is known about how the chemosignals function and which chemical sense is responsible for their mediation. It is known that dominant males exhibit behavioral responses to chemosensory cues (originating from gravid females) alone [7], but it has not yet been shown if these cues are being mediated via olfaction or the other chemosense, gustation. Furthermore, it is also unknown where in the brain these sexually chemosensory signals are processed to elicit appropriate behavioral responses. In this study, we asked which chemosense is mediating the dominant males' recognition of chemosignals from gravid females, and whether the predicted regions of the brain's social behavior network would show differential activation corresponding to the behavioral response of these putative olfactory signals. We hypothesize that olfaction, and not gustation, is the mediating sense for these signals. Further, we hypothesize that, due to the aforementioned regions' extensive degree of connectivity with the brain's primary olfactory processing area and their association with processing social (especially reproductive) behavior, differential activation will occur between gravid and control treatments (as well as between intact versus anosmic treatments) in the Vv, Vs-l, Vs-m, Dp, and nPPa within the SBN.

Methods

Animal Housing

Laboratory raised *A. burtoni* dominant males (standard length (SL): 42.5 ± 2.5 mm) were contained within aquaria held at conditions similar to their natural African habitats, with water at 28° C; pH 8.0; in a 12 h light, 12 h dark cycle, and fed cichlid flakes each morning. These rectangular covered aquaria (50.4 x 25.3 x 30.8 cm) were divided into three compartments (17 x 25. 3 x 30.8 cm for the middle compartment, 16.7 x 25. 3 x 30.8 cm each for the left and right compartments) by solid transparent barriers permanently sealing each compartment off to prevent transfer of chemical signals between compartments (Fig. 1). Sealing was evaluated by filling each compartment with water, adding food coloring to the middle compartment, allowing it to sit overnight and verifying that no dye transfer had occurred between compartments. Each compartment contained water, a layer of gravel at the bottom, an air stone, and a territory refuge/shelter (half of a terracotta pot).

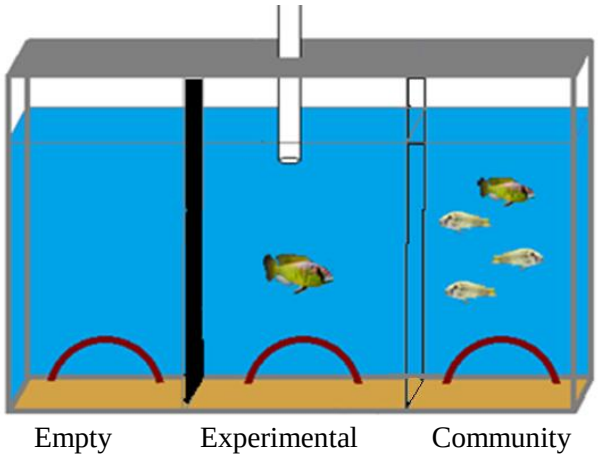

Figure 1. Experimental tank set-up during acclimation. The subject male (middle) was visually isolated from the empty compartment (left). On the day of the experiment, the experimental male was visually separated from the community fish (right) by moving the black barrier over to divide the middle and right compartments.

The middle (experimental) compartment contained the subject fish and the tube mouth of a gravity-feed bottle through which the chemosensory stimulus was administered during behavioral observation. Animal housing and care practices were in accordance with Louisiana State University IACUC regulations.

Flow Rate

A gravity-feed flow system was used to deliver the different chemosensory signals to the central subject male compartment during experiments. The flow rate of stimulus delivery was verified by tests the day before and morning of stimulus delivery to keep the rate within a consistent range (0.325 ± 0.25 L / min) across all trials. First, the tank filter was turned off, and all compartment water levels were leveled to 21 cm from the tank bottom. Gravity-feed bottles were filled with 850 mL of control water and allowed to deliver into the experimental compartment. The time from beginning of stimulus delivery to cessation of flow was recorded to determine flow rate. On the day of the experiment, this test occurred with delivery to the community compartment instead of experimental compartment; filters were turned back on after dissection occurred.

Experimental Conditions

To test how dominant males responded behaviorally to chemosensory cues from sexually-receptive gravid females, four different conditions were tested: Intact Control (olfactory epithelia intact, administered stimulus of normal water), Intact Gravid (olfactory epithelia intact, administered stimulus of water that previously contained gravid females), Anosmic Control (olfactory epithelia ablated, administered stimulus of normal water), and Anosmic Gravid (olfactory epithelia ablated, administered stimulus of water that previously contained gravid females). Five different males were subjected to each set of conditions.

Acclimation Period

Dominant males were identified as being physiologically and behaviorally dominant if they exhibited appropriate coloration and behavior [13] for at least three consecutive days prior to the acclimation period. The acclimation period began when the dominant male was placed into the experimental compartment. To the left of the experimental compartment was an empty compartment of water that was visually obscured from the experimental compartment by a movable black barrier. To the right of the experimental

compartment was a community compartment containing a filter, a smaller dominant male, and three females to simulate a normal social environment and encourage territory establishment by the subject male.

Ablation

To test whether male behavioral responses were mediated by olfaction or gustation, we created anosmic males to eliminate the olfactory sense. Because teleosts contain gustatory receptors throughout their body (mouth, gills, body surface), it is not feasible to eliminate the sense of taste. For anosmic subjects (n = 10), fish were rendered anosmic (~3:30-4:30 p.m.) two to three days minimum after the acclimation period. Since cold temperatures cause anesthesia in fish, anesthesia (for anosmic individuals only) was delivered via submersion in chilled water (~10º C) that also contained a sealed plastic bag containing ice. Fish were considered anesthetized when no movement occurred, even after the water was disturbed for retrieval of the fish. Immediately after, a cauterizer tip was inserted through each naris to ablate the underlying olfactory epithelia. Following ablation, the subject fish was placed into water at the same temperature as lab aquaria and allowed to recover until swimming normally before being placed back into the experimental compartment, where the fish was allowed to recover fully for 48 h before the stimulus was administered.

Stimulus Preparation and Behavioral Recording

On the morning of stimulus delivery (~8:30 - 9:30 a.m.), the subject was fed ~1 flake of cichlid food, and the stimuli were prepared. Gravid stimuli treatments were prepared with four gravid females (identified as such prior to feeding if they had distended abdomens) placed in 850 mL of water. Control stimuli treatments were prepared identically (without any fish). Stimuli were allowed to soak for five hours in a covered bucket with an air stone. Following the soaking period, the air stone and gravid females, if present, were removed from the bucket, and the stimuli were emptied into a gravity feed bottle. The black barrier was removed and placed between the experimental and community compartment such that the subject could observe the empty compartment, but not the community. Thus, the subject males received chemosensory signals alone (either gravid female-soaked or control water), but not visual or other sensory stimuli during trials. The air stones

from all compartments were removed, and the filter was unplugged. A video camera focused on the experimental compartment began recording, and immediately after, the stimulus delivery began. The room containing the tank was vacated of experimenters, and the door was closed, leaving the room undisturbed for 30 min.

Dissection

Following the recording, the room was re-entered, and the recording was stopped. The subject was collected for dissection. Standard length (SL), body mass, and gonadosomatic index (GSI) were recorded. The tail was amputated near the anal pore; then, for a period of a few seconds, blood was taken from the subject into a capillary tube. Then, subjects were immediately sacrificed via decapitation. The dorsal area of the brain case was removed to expose the brain, which was immediately placed for fixation in 4% RNase-free paraformaldehyde (PFA). Blood was centrifuged at 8000 rpm for 10 min to isolate serum, which was removed and measured. The stomach was removed and weighed to subtract from the subject's weight to determine body weight (BW). Testes were removed and weighed to determine gonadal weight (GW). A gonadosomatic index (GSI), was determined using the equation: $GSI = (GW/BW) \cdot 100$. Testes and serum were then stored at -80° C. The experimental compartment was drained and refilled between trials to prevent cross-contamination.

Sectioning

Following removal, brains were stored overnight at 4° C. Following the fixation period, the brain was transferred to 1X RNase-free PBS (phosphate-buffered saline) in refrigeration, where it remained for a minimum of one day. For extended storage in PBS, brains were placed in fresh PBS twice a week. Following the PBS rinse period, the subject's brain was cryoprotected in 30% sucrose. The brain was then sectioned with a cryostat at 20 μm sections, collected on charged slides, and stored at -80° C.

Colorimetric in situ *Hybridization*

To determine which neurons in the brain were activated, we used *in situ* hybridization to label the immediate early gene *c-fos* as a proxy for neural activation. An *in situ* hybridization protocol was adapted from [23] with riboprobes for *c-fos*. Gene-specific riboprobes were constructed with digoxigenin (DIG) - labeled uracil. A hydrophobic barrier was drawn on the edges of each slide using an ImmEdge Pen. Sections were rehydrated with washes in PBS, fixed with 4% PFA, rinsed in PBS, then permeabilized with proteinase K (10 μg/mL). Sections were washed with PBS then fixed again, followed by a PBS rinse. Then, 0.25% acetic anhydride in 0.1 M triethanolamine-HCL (pH 8.0) was applied. After another PBS rinse, pre-hybridization occurred in hybridization solution in a sealed humidified chamber at 60-65˚C for 3 h. Subsequently, the solution was replaced by hybridization buffer containing probes for *c-fos.* Slides were covered with HybriSlip covers and left to hybridize overnight at 60-65˚C in sealed humidified chambers. Then, at this same temperature, sections were washed in 2X SSC in 50% Formamide with 0.1% Tween-20, then in a 1:1 mixture of 2X SSC and maleic acid buffer with 0.1% Tween-20 (MABT). MABT washes were performed again at room temperature. Then, blocking occurred in incubation with MABT and 2% bovine serum albumin (BSA) for 3 h. Slides were then incubated with alkaline phosphatase (AP) anti-DIG fragments at 1:5000 in MABT 2% BSA overnight at 4˚C in a sealed humidified chamber. After antibody incubation, slides were washed in MABT, then rinsed in AP buffer, and then developed with NBT/BCIP substrate at 37˚ C in darkness for 1-3 h. Development was stopped with a PBS rinse. Slides were then fixed in 4% PFA, then washed in PBS, then coverslipped.

Cell Quantifications

Quantification of activated cells was performed in the Vv, nPPa, Dp, Vs-l, and Vs-m regions. For Vv and nPPa, each section was overlaid with a grid of squares, each 225 μm^2, and three squares within the region of question were chosen at random. Cells were counted at 200X magnification, and cell density per section was determined by dividing total cell count of three squares by 675 μm^2. For Dp, Vs-l, and Vs-m, grid squares were each 2500 μm^2, counted at 100X magnification, and density determined by dividing total cell

count by 7500 μm^2. Cells were counted if activated, and activation was determined as having at least 3/4 of the cell border stained with some internal staining. Four sections were quantified per brain and then averaged to obtain a single value per individual fish.

Behavioral Scoring

Behavioral recordings were later scored for two behaviors, erratic swims and pot entries. Erraticism was defined as multidirectional "repetitively up and down" vertical swimming behavior involving full-body undulations at high speed while facing the tank walls for a minimum of 2 s. Separate swims were defined as having an interval of at least 1 s of non-erratic behavior between them. Pot entries were defined as instances when the subject male was at least half of one body length entering the pot refuge. Duration was also recorded for erratic swims and time spent under refuge (pot duration). Aside from the full 30 min recording, erratic swims were also analyzed using only the first and last 10 minutes of the video. Latency to first erratic swim was also analyzed.

DASPEI Staining

One subject was used for DASPEI (2-[4-(dimethylamino)styryl]-*N*-ethylpyridinium iodine) staining for verification of epithelial cauterization to generate anosmia. DASPEI is a vital and voltage-sensitive dye that is capable of preferentially labeling sensory neurons, including those found within the nasal epithelium, and can therefore be used to indicate the presence and structure of olfactory tissue [24]. The same experimental protocol regarding the subject was observed up until stimulus delivery, which did not occur. Instead, the subject was placed into a DASPEI solution of 0.008% in darkness for five minutes. Following staining, the subject was sacrificed via placement in a beaker containing water and ~2 g of benzocaine. The subject was then taken for examination of cauterization wounds under fluorescence microscopy; images were taken at 2000 ms exposure.

Statistical Analysis

SigmaPlot 12.3 was used to create graphs and run statistical analysis. Two-way ANOVA (Test Factor 1: Intact/Anosmia; Test Factor 2: Control/Gravid) was performed ($\alpha = 0.05$) to determine significance for behavioral data. Two-tailed t-tests ($\alpha = 0.05$) were used to determine significance for *c-fos* activation of intact groups. For data sets corresponding to erratic swims and erratic duration, both for the first ten minutes, as well as pot entries, the normality assumption of the ANOVA was violated, so log transformations were utilized to achieve normality for erratic swims of the first ten minutes and pot entries. Although transformations (log, ln, and square root) were also attempted for erratic duration of the first ten minutes, normality was still not achieved, so these data were tested untransformed and in violation of the normality assumption.

Results

Behavior: Frequency of Erratic Swims

To test whether dominant males would exhibit stimulus-dependent as well as anosmia-dependent behavioral alteration, males (either possessing intact or ablated nasal epithelia) were exposed to chemosensory stimuli that either did (gravid stimulus) or did not (control stimulus) undergo a soaking period containing gravid females. For the full 30-minute observation period, no significant interaction was observed regarding frequency of erratic swims between the two analysis factors (ANOVA: $P = 0.169$), but males exposed to the gravid stimulus swam erratically significantly more often than males exposed to control water (ANOVA: $P = 0.004$; Fig. 2A). For the first 10 minutes of the recording, no significant interaction was observed between the two analysis factors (ANOVA: $P = 0.094$), but males exposed to the gravid stimulus swam erratically significantly more often than those exposed to control water (ANOVA: $P = 0.003$), and intact males swam erratically significantly more often than anosmic males (ANOVA: $P = 0.037$; Fig. 2B). For the last 10 minutes of the recording, no significant interaction was observed between the two analysis

factors (ANOVA: P = 0.408), but males exposed to the gravid stimulus swam erratically significantly more often than those exposed to control water (ANOVA: P = 0.040; Fig. 2C).

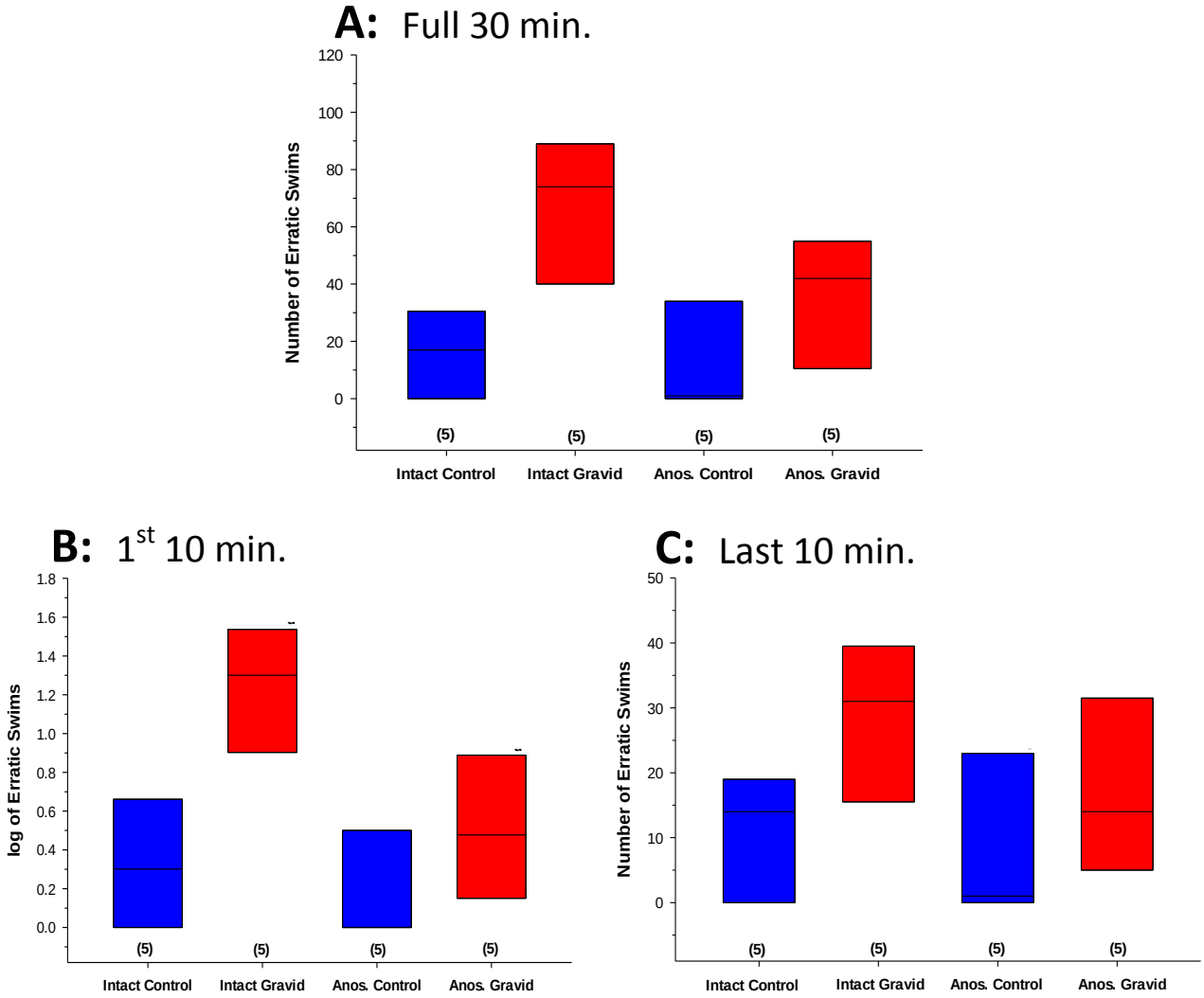

Figure 2: Erratic swimming behavior of subject males exposed to chemosensory signals. (A) Over the 30-min duration of the experiment, dominant male *A. burtoni* swam erratically significantly more often when presented with gravid vs. control stimulus. (B) Over the first 10 min of recording, males swam erratically significantly more often when presented with gravid vs. control stimulus, and anosmic individuals swam erratically significantly less often than intact males. (C) Over the last 10 min, males swam erratically significantly more often when presented with gravid vs. control stimulus. Sample size for each group is shown beneath the corresponding bar. Groups that received the control stimulus groups are in blue, and those that received the gravid stimulus are in red.

Behavior: Erratic Swim Duration

With regard to time spent swimming erratically, for the full 30-min observation period, no significant interaction was observed between the two test factors (ANOVA: $P = 0.397$), but males exposed to the gravid stimulus swam erratically for a significantly longer time than those exposed to control water ($P = 0.013$; Fig. 3A). For the first 10 minutes, no significant interaction was observed between the two test factors (ANOVA: $P = 0.067$), but males exposed to the gravid stimulus swam erratically for a significantly longer time than those exposed to control water ($P = 0.020$), and intact males swam erratically for a significantly longer time than anosmic males ($P = 0.045$; Fig 5B). For the last 10 minutes, no significant interaction was observed between the two test factors (ANOVA: $P = 0.587$), but males exposed to the gravid stimulus swam erratically for a significantly longer time than those exposed to control water ($P = 0.027$; Fig 3C).

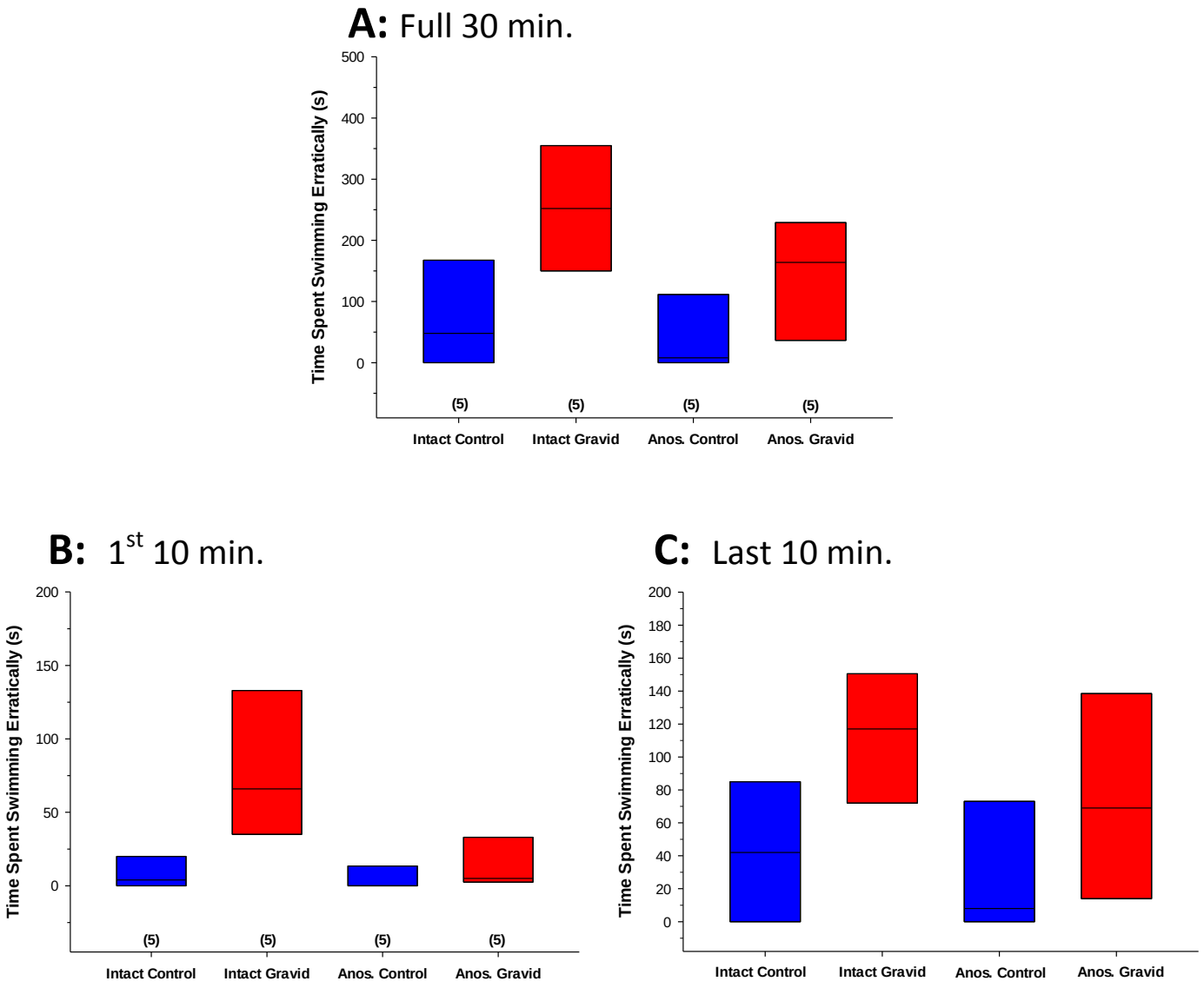

Figure 3: Duration of erratic swimming behavior of subject males exposed to chemosensory signals. (A) Over the 30-min. duration of the experiment, dominant male *A. burtoni* swam erratically for a significantly longer time when presented with gravid vs. control stimulus. (B) Over the first 10-min. of recording, males swam erratically for a significantly longer time when presented with gravid vs. control stimulus, and anosmic males swam erratically for a significantly shorter time than intact males. (C) Over the last 10 min., males swam erratically significantly more often when presented with gravid vs. control stimulus. Sample size for each group is shown beneath the corresponding bar. Groups that received the control stimulus groups are in blue, and those that received the gravid stimulus are in red.

Behavior: Latency to First Erratic Swim

With regard to the time before the first erratic swim, no significant interaction between test factors was observed (ANOVA, P = 0.818), but males exposed to control water had a significantly longer latency than those exposed to the gravid stimulus (P = 0.006; Figure 6).

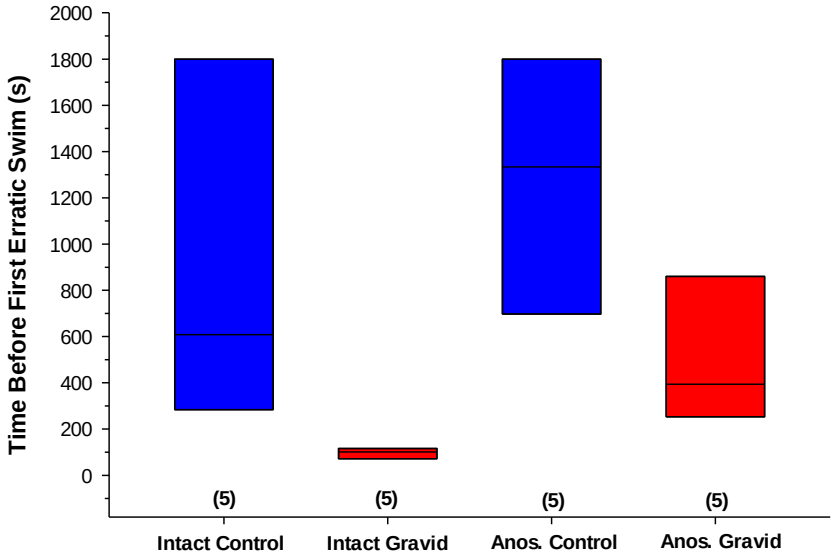

Figure 6: Latency to erratic swimming behavior in subject males exposed to chemosensory signals. Gravid stimulus caused decreased latency before first erratic swim. Sample size for each group is shown beneath the corresponding bar. Groups that received the control stimulus groups are in blue, and those that received the gravid stimulus are in red.

Behavior: Frequency of Pot Entry

No significant interactions nor main effects were observed for frequency of pot entry (ANOVA, P = 0.652; Fig. 4).

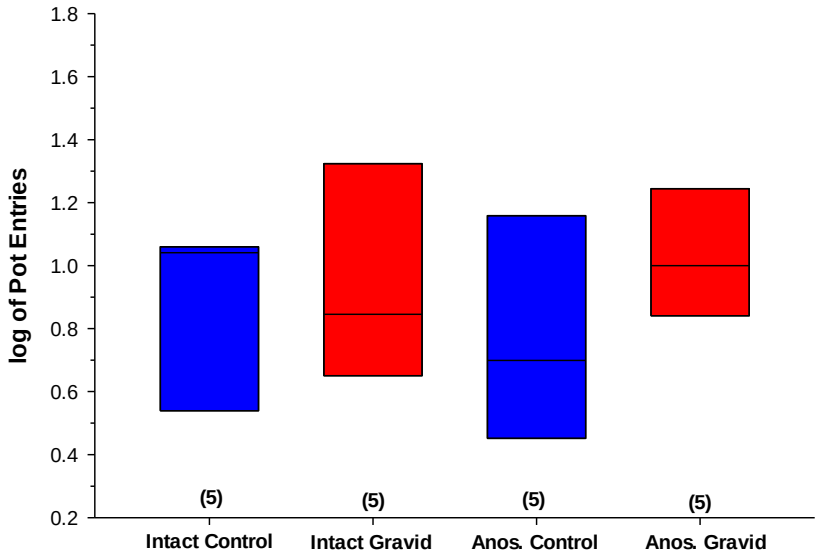

Figure 4: Pot entrances of subject males exposed to chemosensory signals. Neither stimulus type nor epithelial state affected frequency of pot entries. Sample size for each group is shown beneath the corresponding bar. Groups that received the control stimulus groups are in blue, and those that received the gravid stimulus are in red.

Behavior: Pot Duration

No significant interaction nor main effects were observed for pot duration (ANOVA, P = 0.364; Fig. 5).

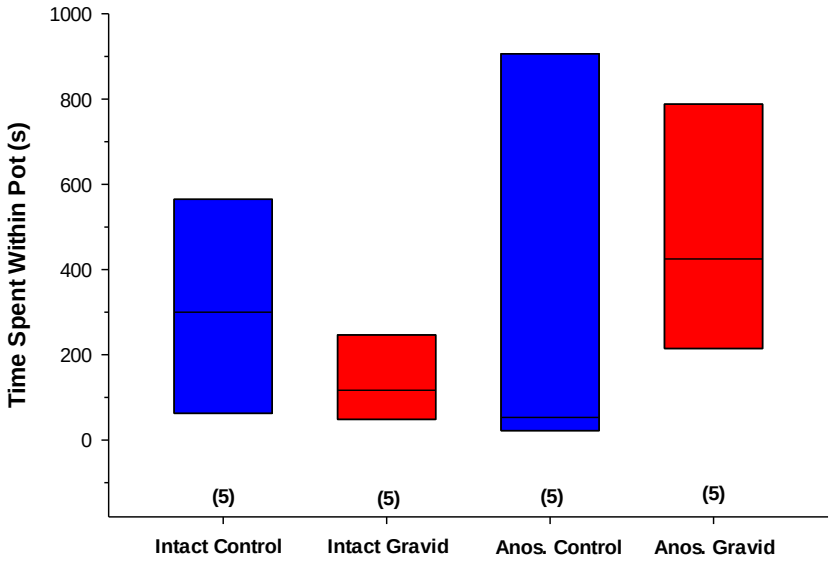

Figure 5: Duration of period subject males exposed to chemosensory signals spent within a pot refuge. Neither stimulus type nor epithelial state affected time spent within pots. Sample size for each group is shown beneath the corresponding bar. Groups that received the control stimulus groups are in blue, and those that received the gravid stimulus are in red.

DASPEI Stain

To verify that olfactory epithelia were fully ablated and remained so by the time of the trial, we submerged an ablated dominant male in DASPEI solution (0.008%) and examined the cauterization wound under fluorescence microscopy. DASPEI stain revealed no epithelial structure within the cauterization wound (Fig. 7).

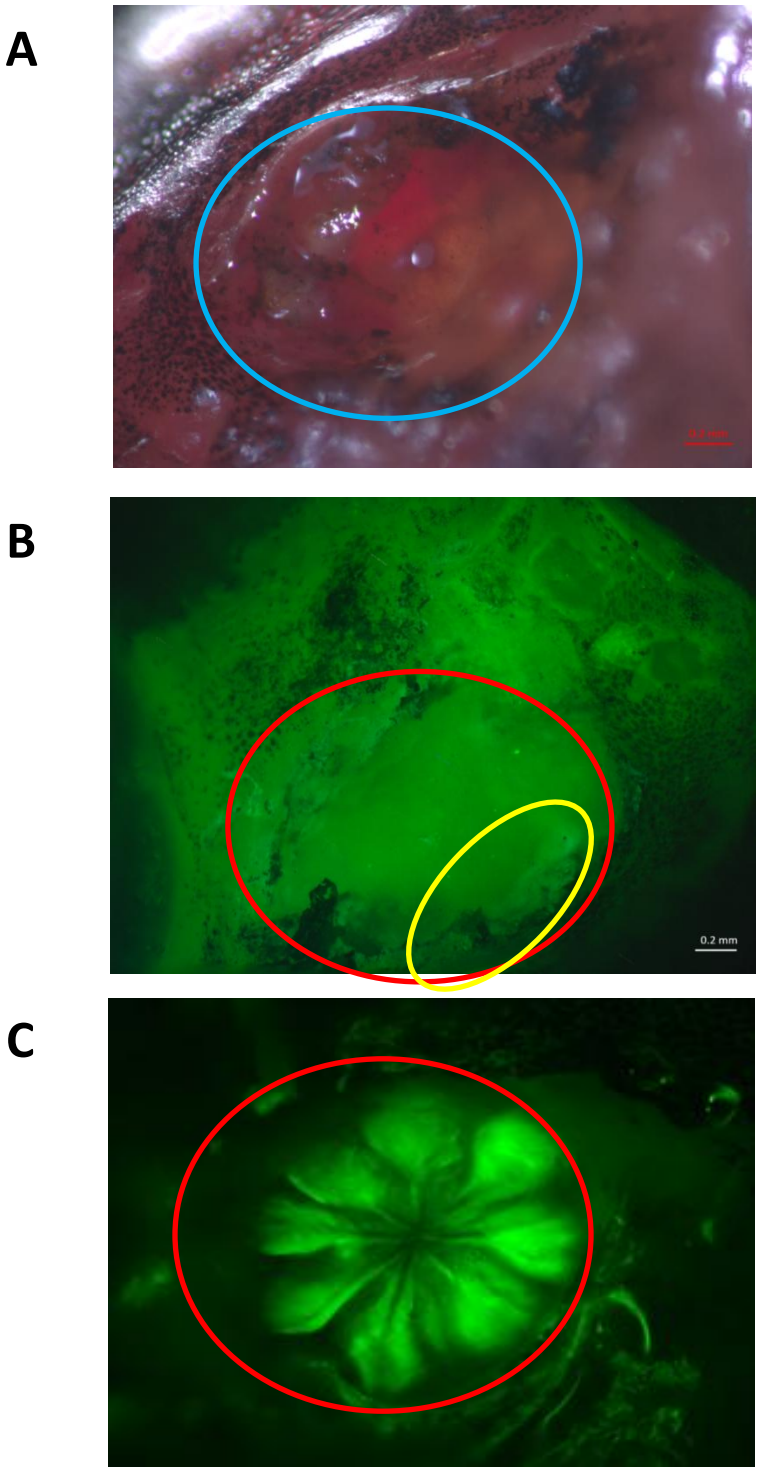

Figure 7: Olfactory epithelia of *A. burtoni* with DASPEI stain. Cauterization wound after DASPEI stain shows no epithelial structure. (A) Cauterization wound (outlined in blue) under brightfield. (B) Cauterization wound (outlined in red) under fluorescence. Possible scar tissue fluoresces in lower right area (outlined in yellow). (C) Intact *A. burtoni* olfactory epithelia showing rosette structure (outlined in red); photograph of intact epithelia derived from a specimen under a different experimental protocol within a separate project.

Neural Activation: Ventral Telencephalon

To test for neural correlation of behavioral response to chemosensory cues, cells in brains of subjects within the behavioral trials were quantified for *c-fos* activation as represented by color development from *in situ* hybridization (Fig 8). Anosmic groups are shown in boxplots but were not tested statistically because of insufficient sample size. No significant difference was observed with regard to active cell density between intact groups that were exposed to gravid stimulus or control water in the Vv (t-test, $P = 0.304$, Fig. 9A), Vs-l ($P = 0.858$, Fig. 9B), or Vs-m ($P = 0.399$, Fig 9C).

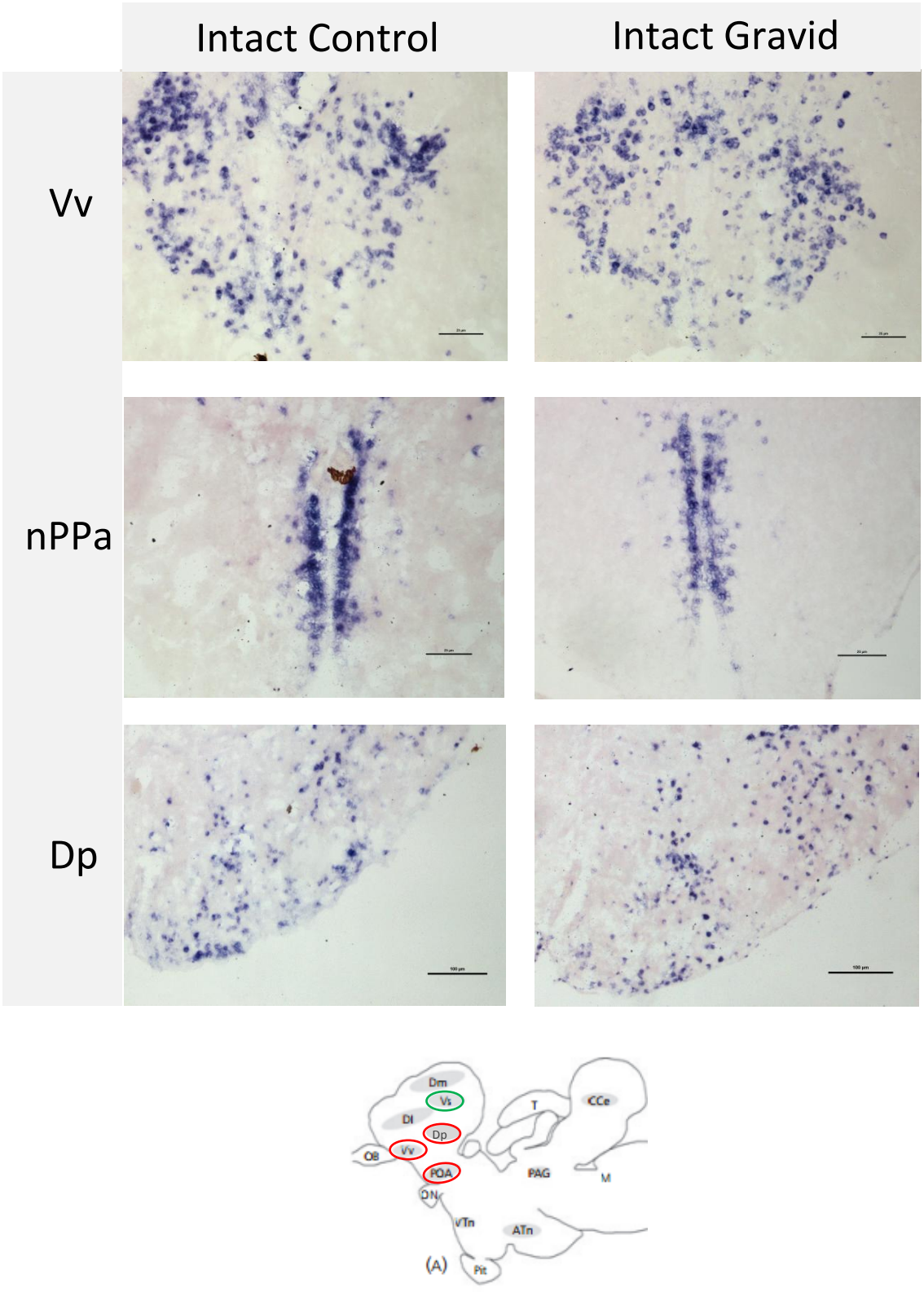

Figure 8: Colorimetric *c-fos* staining in the Vv, Dp, and nPPa of intact males. Stimulus treatment indicated at the top of each column. Vv and nPPa shown at 200X magnification, Dp at 100X. Beneath the photographs is a diagram adopted from Maruska, et al. 2012, of a sagittal view of the *A. burtoni* brain with photographed regions outlined in red. Vs (green) was quantified but is not shown in photographs.

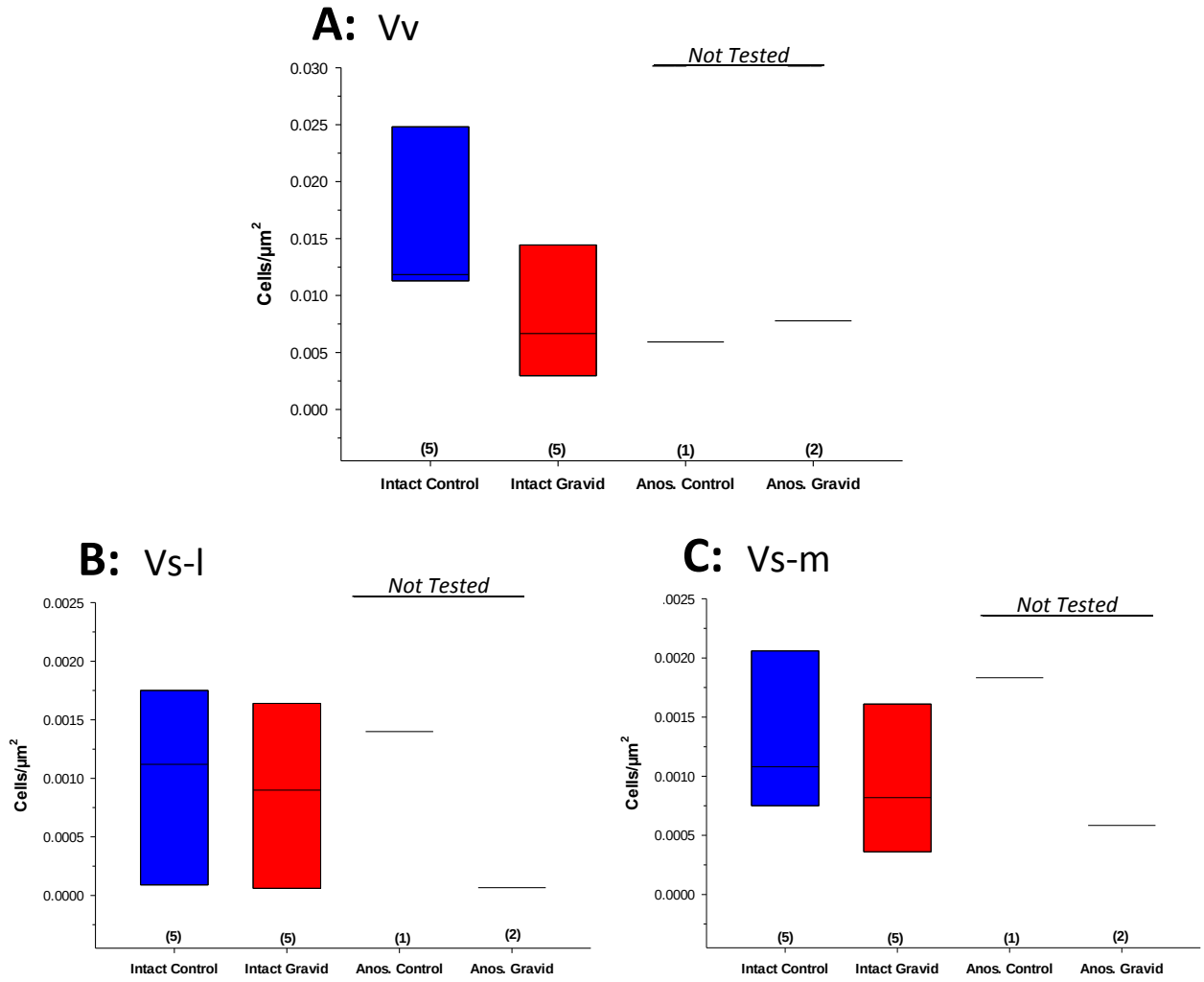

Figure 9: Neural activation in regions of the ventral telencephalon of subject males exposed to chemosensory signals. Adding gravid stimulus did not cause brain activation in the ventral telencephalon regions to be higher than in control individuals. Intact dominant male *A. burtoni* demonstrate no significance for cellular activation with regard to chemosensory stimulus in the: (A) Vv, (B) Vs-l, or (C) Vs-m. Horizontal lines in "not tested" groups indicate medians. Sample size for each group is shown beneath the corresponding bar. Groups that received the control stimulus groups are in blue, and those that received the gravid stimulus are in red.

Neural Activation: nPPa

Anosmic groups are shown but were not tested statistically because of insufficient sample size. No significant difference was observed with regard to active cell density between intact groups that were exposed to gravid stimulus or control water in the nPPa (t-test, P = 0.271, Fig. 11).

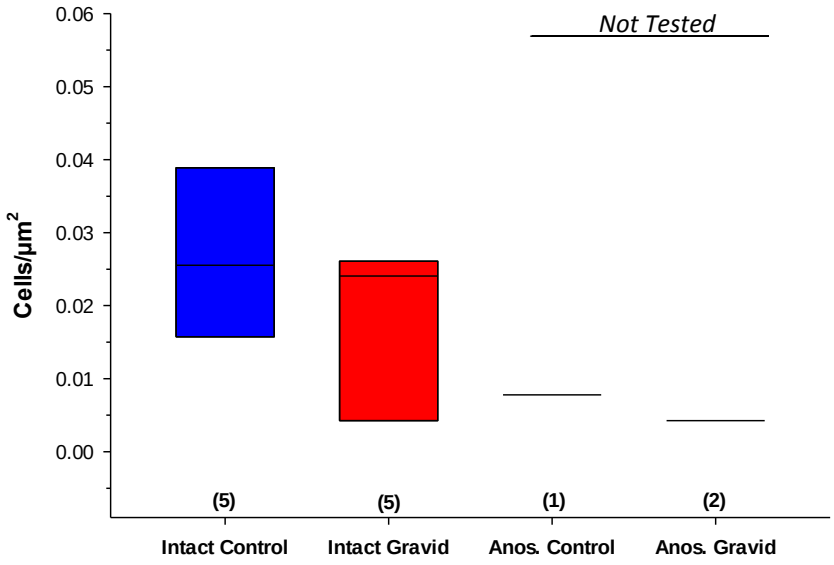

Figure 11: Neural activation in regions of the nPPa of subject males exposed to chemosensory signals. Adding gravid stimulus does not cause brain activation in the nPPa to be higher than in control individuals. Intact dominant male *A. burtoni* demonstrate no significance for cellular activation with regard to chemosensory stimulus in the nPPa (t-test, P = 0.470). Horizontal lines in "not tested" groups indicate medians. Sample size for each group is shown beneath the corresponding bar. The group that received the control stimulus is in blue, and those that received the gravid stimulus are in red.

Neural Activation: Dp

Anosmic groups are shown but were not tested statistically because of insufficient sample size. No significant difference was observed with regard to active cell density between intact groups that were exposed to gravid stimulus or control water in the Dp (t-test, P = 0.470, Fig. 10).

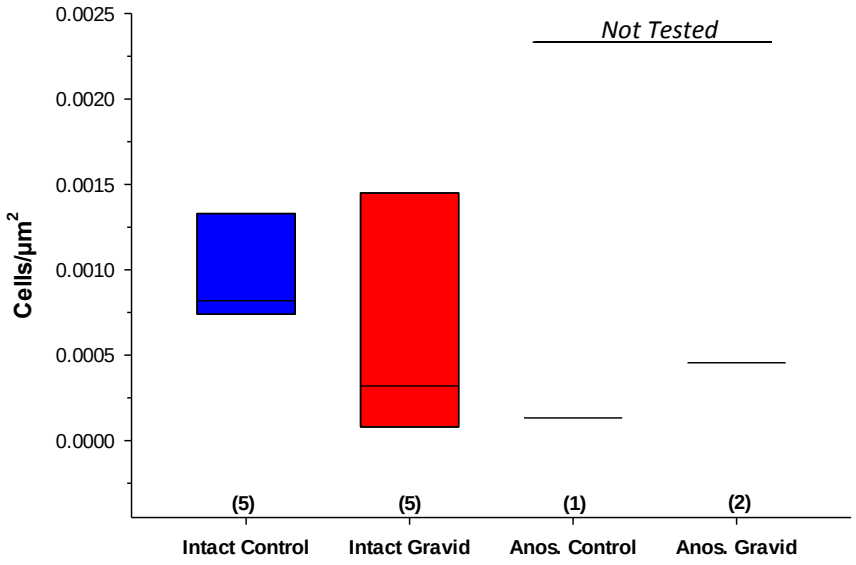

Figure 10: Neural activation in regions of the Dp of subject males exposed to chemosensory signals. Adding gravid stimulus did not cause brain activation in the Dp to be higher than in control individuals. Intact dominant male *A. burtoni* demonstrate no significance for cellular activation with regard to chemosensory stimulus in the Dp. Horizontal lines in "not tested" groups indicate medians. Sample size for each group is shown beneath the corresponding bar. The group that received the control stimulus is in blue, and those that received the gravid stimulus are in red.

Discussion/Conclusion

Our results demonstrate that dominant male *A. burtoni* exhibit increased behavioral responses, in the form of erratic swimming, to gravid female chemosensory cues compared to control males (Figs. 2, 3, and 6). Furthermore, our results also demonstrate that anosmia decreases the frequency and duration of the erratic swim response, but only within a limited time period (Figures 2B and 3B). The behavioral parameter of staying within the pot refuge did not demonstrate a clear effect of either stimulus or anosmia for frequency or duration (Figs. 4 and 5). Lack of olfactory epithelial structure in the DASPEI stain suggests that ablation procedures were indeed effective (Fig. 7). Our results for brain activation do not show a different response elicited by gravid chemosensory cues alone (compared to control) in various regions of the social behavior network (Figs. 8-11).

Behavior: Erratic Swimming

Our results that demonstrate higher levels of erratic swimming in males exposed to gravid female-soaked water compared to control water support our hypothesis that these chemosensory cues alone can influence male behavior. As expected, our data support earlier studies [6, 7] showing that dominant males exhibit behavioral responses to chemosensory cues from gravid females, although the nature of these responses differ. Although the previous studies showed response in the absence of visual stimulation from the source of the chemosensory cues, it is worth noting that in these cases, the dominant male fish did have visual stimulation in the form of young blind *Tilapia mariae* [6, 7]. Although these *Tilapia* sp. were behaviorally hindered because of lack of sight, the dominant male *A. burtoni* clearly received some visual stimulation from these fish (even though the administered chemosensory cues were derived from conspecifics), as they performed a suite of behaviors associated with courting and reproduction [6], behaviors which require some form of visual stimulus to elicit [2]. The lack of visual cues in our experimental design necessitates the use of erratic swimming behavior as a proxy for detecting reproductive interest without actually performing reproductive behavior used in courtship and spawning. Our results show that there are significant differences in both erratic swimming behavior as well as duration between control versus gravid treatment at all examined time frames. Furthermore, it has been demonstrated that the urination rates that increased in the presence of a receptive female are observed at even higher rates when the male had no chemosensory connection with the female, suggesting that the males may be attempting to elicit chemosensory cues indicative of stimulation from the female [2]. When visual cues are given, courtship behavior by dominant males shows an approximately tenfold decrease in situations lacking chemosensory information compared to those with both visual and chemosensory input [2]. In other teleosts, it has been shown that chemosensory cues deriving from reproductively receptive females increase both male reproductive behavior and physiology, such as increased milt volume and sperm motility [25], characteristics associated with the social ascent and accompanying increase in reproductive capacity of male *A. burtoni* [26, 27] as well as members of other taxa, including mammals [1].

Differences in response based on intact versus anosmic olfactory epithelia were also present, suggesting the use of olfaction for this pheromonal communication. However, these differences were only seen during the first 10 minutes of the behavioral recording. When the video is examined in its entirety, the differences between intact versus anosmic groups seem to disappear, while the control versus gravid groups remain different. We suggest that this change is due in part to a masking effect arising in the later portions of the video. Administration of the chemical stimulus into the tank is complete at less than three minutes, which means that the subject remains without any new social stimuli once its epithelia are desensitized to the cue [28]. Since the concentration of chemical cues elicited by the gravid females during the soaking period was not quantified, no estimate of reliable accuracy can suggest when the subject fish becomes desensitized, unless behavioral response itself is considered. When only the last 10 minutes of the video are examined, the anosmic and intact groups are statistically similar (although the difference between gravid and control does remain). It is also worth noting that our sample sizes (n = 5 for each group) are relatively small for a behavioral study. A common feature of behavioral studies is intrinsic interindividual variation, or behavioral syndromes [29], that have been acknowledged as prominent in other studies on social cichlids originating from Lake Tanganyika [30-32]. The intrinsic behavioral variation features more prominently in our results because of small sample size, although the general trend consistently shows the intact gravid group's level of behavioral response rising above the others. It is possible that, with increased samples, this trend will become more distinct. Furthermore, the control water treatment was simply taken from the laboratory reservoir of filtered water that serves as the source of water in all of the laboratory fish tanks; a *post hoc* evaluation of this practice revealed that there are trace concentrations (that vary temporally) of unknown substances in the water that are not filtered out. This implies that, for the Intact Control group, there may be some behavioral variation because of the particular levels of solutes within the stimulus water source upon the day of trial. Future tests using water filtered by reverse osmosis as a control stimulus will help resolve this issue.

Behavior: Latency to First Erratic Swim

Groups receiving gravid stimulus had a shorter latency than groups receiving control treatments (Fig. 8). Although this is not entirely surprising, it is unexpected that the intact groups do not also have a shorter latency, when compared to the anosmics. However, the data for the Intact Gravid group clearly appears to exhibit a much shorter and less variable latency than the other three groups, which is indicative of the dominant males' immediate sensitivity and response to the female chemosensory cues. Even with our small data set of behavioral parameters, this group is showing very little variation in their tendency to respond quickly to the administered stimulus, unlike any of the other groups. However, the intact control group has a considerably large amount of variation (possibly due, in part, to the aforementioned variation in the stimulus water), which could mask the differences from the anosmic groups.

Behavior: Pot Entry and Duration

We found no differences in frequency of entry into nor duration within pots (Figs. 6 and 7), which is not entirely surprising. Pot entry and duration were originally included in this study as another proxy for courtship behavior, as the compartment pot comprises the dominant male's central territory, and a courting pair of fish will enter a pot (or similar structure in a natural setting) to spawn; pot entries can exemplify "leading" behavior of *A. burtoni* in which a dominant male attempts to lead a female into the pot to spawn [2, 6, 7, 13]. However, the pot also serves as a general refuge or protected area; therefore, fish may enter the pot because of high stress levels and/or during an attempt to escape a perceived threat. Fish were noted to enter and stay within the pot for extended periods of time during stressful situations, such as at the beginning of the acclimation period, during recovery following ablation, and invasive human presence (unpublished observations). Furthermore, fish in these conditions also possessed vertical black bars along the length of the body, a coloration associated with stress in these species [16]. However, the function of the two acclimation periods was to allow for the stress response as a result of trauma from being moved into the tank or ablated, and fish with severe stress reactions to recording were not included in the data set, so intense stress reaction is not a likely cause of the lack of difference. These results suggest that pot entries, although intended as a simulation of leading behavior, require visual cues like true reproductive behavior to be performed in a sexual

context. It is intuitive that a dominant male would not perform leading behavior if there is no female to lead. Digging in the substrate beneath territory pots to entice female entry is another reproductive behavior performed by dominant males, but this behavior was never observed within any recording. This is also probably due to the lack of visual stimulation. Considering pot duration (Fig. 7), although no differences were observed, anosmic groups appeared to trend higher than the intact groups, possibly alluding to some residual stress from the ablation procedure. It is also intuitive that, since the anosmic fish cannot smell (and therefore are not receiving any social stimulus), and there is no food in the tank to be detected via gustation, there is little to motivate them to exit the safety of the pot.

DASPEI Stain

We found a lack of nasal epithelial structure in the cauterization wound (Fig. 9), indicating that the ablation procedure was indeed effective. The olfactory epithelia are rosette-shaped [33] (Fig. 7C), so lack of this morphological organization suggests absence of the epithelium, and therefore absence of olfactory function. Although the olfactory epithelium is capable of regeneration to some extent [34], it appears that, possibly because of thorough ablation or a recovery duration too short to allow for regrowth, epithelial structure remains nonexistent after the recovery period following ablation. It is worth noting that there was some abnormal fluorescence in the image (Fig. 7B, yellow outline), and the cause of this is unknown. It is possible that the fluorescence results from some residual epithelial tissue, but because of the lack of any apparent shape or formation, it is most likely scar tissue. Again, low sample sizes are an issue here, and more stains are needed to substantiate evidence that the ablations are successful.

The anosmic fish do exhibit some (albeit to a relatively smaller) degree of behavioral response to the gravid stimulus that is not present during the control stimulus, suggesting the possibility that some chemosensory information is being received via gustation. Gustatory cells in teleosts are capable of responding to a variety of different chemicals, including amino acids, sugars, carboxylic acids, bile salts, and carbon dioxide, but are usually not responsive to sexual pheromones and generally lack a role in reproductive behavior [12]. Gustation is usually associated with feeding behavior, and, to our knowledge, no study to date

has implicated a role for gustation in social behavior. Therefore, it is possible that the anosmic males' responses to the gravid stimulus may be due to non-reproductive chemicals present in the stimulus. Since the gravid stimulus composition was never analyzed or evaluated, it is unknown what potential chemicals could have been present and caused the observed responses. It is apparent that the gravid stimulus water contained higher carbon dioxide content because of the respiration of the females during the soaking period and the early stages of decomposition of their feces, which contain bile salts, known to be potent signals in some teleosts via olfaction as well as gustation [12]. Furthermore, the stimulus water likely contained some of the gravid females' mucus, a secretion known in some species of cichlids to contain free amino acids and other bioactive compounds [35]. Also, the erratic swimming behavior, although demonstrated to be significantly more frequent after administration of the gravid stimulus compared to control, remains a functional mystery and is not true reproductive behavior. That being said, it is unknown what biological motives there are for performing this behavior, and it may be that it is a generalized response that occurs in response to a variety of chemical stimuli, not only those reproductive in nature.

Neural Activation

With regard to brain activation, our results do not support our hypothesis, as there is no difference among any statistically testable group within our data set, suggesting an absence of stimulus-dependent differential activation in the tested regions because of stimulus administration in the experimental paradigm. Again, the regions we selected were affiliated with olfaction and reproduction via known connections in the teleost forebrain to the olfactory bulb, and association with reproductive behavior [21, 22]. However, in the current paradigm, the lack of visual stimuli eliminates the possibility of true reproductive behavior characteristic of actual courting and spawning. No such behavior was observed in any of the behavioral recordings. Although these fish demonstrated behavioral responses when exposed to the gravid stimulus, indicating reception of the stimulus, the behavioral responses were not true reproductive behavior (since they are not performed with a female during courtship or spawning) and therefore apparently not differentially mediated via these regions. Therefore, since there is no reproductive behavior occurring in any trials, and

therefore no difference in reproductive behavior across the different experimental groups, despite the differences in epithelial state and stimulus, it follows that the neural correlates would also show no difference within the brain. Had there been more activation as a result of one treatment, it is expected that the group under that treatment would have also exhibited increased reproductive behavior.

Although chemosignals can and are released based on social context, once they are added into the transfer medium (in this case, the surrounding water), they may remain there for a time even if the signaler organism is not present or move away from the signaler due to currents in the medium. If chemosignals alone triggered a brain activation pattern inciting reproductive behavior, dominant males would respond with reproductive behavior every time a sexual odorant was sporadically detected, regardless of the female's accessibility. Such a situation seems energetically and temporally wasteful compared to one in which a male only courts after receiving the visual (and guaranteed) stimulus. In this way, our results for brain activation support our results for behavior. We did expect that the anosmic brains would show less activation in the absence of chemical stimulation, but not much conclusive information is offered by the limited anosmic results. Although the anosmic brain data was not statistically tested, one message the data may suggest is that in the Dp, a region whose primary function involves the reception of olfactory cues, the anosmic groups vaguely appear to trend lower than the intact groups, which is expected in the absence of olfactory sensory information. Therefore, based on these results, the Vv, Vs, Dp, and nPPa still remain possible regions worth considering for mediating olfactory cues to produce appropriate responses in settings coupled with visual stimuli, and this claim is still supported by what is known about these regions, including reception of olfactory-related information via known connections to the teleost olfactory bulb and prominence within the SBN as a nucleus mediating sexually relevant behavior [21, 22, 35].

Future Directions

The optimal future action to take regarding this project would be to increase sample sizes. Ideally, this would decrease the variation present in behavioral data, as well as provide more sound evidence that the ablation is successfully causing anosmia. It is interesting to note that across the different time ranges, the intact gravid group trends the highest on the graph for both amount of erratic swims as well as duration of erratic swimming, with the other three groups beneath it, which is expected. This latent trend may become more defined in the future if variation were to decrease. It would be beneficial to perform scoring of the behavioral recordings blind to eliminate influence of bias from knowledge of the applied treatments. The experimental protocol could be expanded to adopt another experimental group, sham fish, who would be cauterized at an area other than the olfactory epithelia, thus possessing all of the effects of cauterizing trauma without affecting olfactory function, to verify that the process and stress of cauterization itself is not altering the behavioral results. Furthermore, another treatment using fish of no particular social/reproductive status (such as juveniles) in place of gravid females would provide information regarding how much of the behavioral response we observed was due to "organismic chemistry" vs. conspecifics of a particular reproductive state. To eliminate the effects of variation in the control treatments used here, using water filtered by reverse osmosis would provide a more reliable control treatment. If gustation is being used to register non-reproductive substances emitted by the gravid females, trials with non-gravid females would be useful in separating the influence of reproductive odorants from gustatory signals. It would also be beneficial to quantify regions associated with gustation, which will likely show differential activation due to the irrelevance of reproductive behavior to gustatory function. Finally, adding yet another group, one with a visual element, is a currently ongoing addition to the chemosensory stimulus project. This will allow for the observation and analysis of true reproductive behavior, eliminating the need for a proxy to estimate responses to stimuli. This would also imply an observable link to brain activation in the regions mentioned here, which should be reexamined in the visual stimulus trials. Adding a visual element will also allow for the interpretation of unimodal sensory cues from vision alone rather than solely via a chemosensory branch. This will also allow for the comparison of only chemical stimuli versus only visual stimuli versus both stimuli

integrated together, for a more complete understanding of the complex interaction between these sensory systems during processing external stimuli and internal state to exhibit contextually appropriate behavior.

Acknowledgements

I would like to thank Karen Field for her highly invaluable advice, guidance, intelligence, and perseverance, all provided from the very beginning; Dr. Karen Maruska for her generosity welcoming me into this lab group and the enormous amount of keen insight and patience provided since; Julie Butler for her photograph (Fig. 7C) and the many useful pieces of advice she also has offered; Dr. Kyle Harms for his inexhaustible thoughtfulness and counsel, especially when it was needed most; Dr. Ann Holmes and Dr. Patricia Moroney for their considerable amount of input, time, and service on the defense committee; Whitney Gentry Walkowski and Danielle Porter for providing still more guidance; Kara Johnson for her unfailing helpfulness and cooperation; Charly Brooks for his free assistance with data collection and attention to detail; Daniel Vilchez for his support and scheduling abilities; David Roberts for his assistance in general and with defense preparation; Charles deBoisblanc also for his assistance with defense preparation, and the Maruska Lab members for their help.

References

1. **Swaney, W. T. and Keverne, E. B.** (2009). The evolution of pheromonal communication. Behav. Brain. Res. 200, 239-247.
2. **Maruska, K.P. and Fernald, R.D.** (2012) Contextual chemosensory urine signaling in an African cichlid fish. J. Exp. Biol. 215: 68–74.
3. **Sorensen, P**. (1992). Hormonally derived sex pheromones in goldfish: a model for understanding the evolution sex pheromone systems in fish. Biol. Bull. 183, 173-177.

4. **Rosenthal, G. G., Fitzsimmons, J. N., Woods, K. U., Gerlach, G. and Fisher, H. S.** (2011). Tactical release of a sexually-selected pheromone in a swordtail fish. PLoS ONE 6, e16994.
5. **Dulac, C. and Torello, A. T.** (2003). Molecular detection of pheromone signals in mammals: from genes to behaviour. Nat. Rev. Neurosci. 4, 551-562.
6. **Crapon de Caprona, M. D**. (1980). Olfactory communication in a cichlid fish, Haplochromis burtoni. Z. Tierpsychol. 52, 113-134.
7. **Crapon de Caprona, M. D.** (1974). The effect of chemical stimuli from conspecifics on the behavior of Haplochromis burtoni (Cichlidae, Pisces). Experientia 30, 1394-1395.
8. **Van Staaden, M. J. and Smith, A. R.** (2011). Cutting the Gordian knot: complex signaling in African cichlids is more than multimodal. Curr. Zool. 57, 237-252.
9. **Maruska, K.P., Ung, U., and R.D. Fernald**. (2012). The African cichlid fish *Astatotilapia burtoni* uses acoustic communication for reproduction: sound production, hearing, and behavioral significance. *PLoS One 7(5): e37612.*
10. **Fernald, R. D, Liebman P. A** (1980) Visual receptor pigments in the African cichlid fish, Haplochromis burtoni. Vision Res 20: 857–864.
11. **Insel, T.R.** (2010). The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior. Neuron 65: 768-779.
12. **Hara, T.J.** (1994). Olfaction and gustation in fish: an overview. Acta Physiological Scandinavica. 152 (2): 207-
13. **Fernald, R. D. and Hirata, N. R.** (1977). Field study of Haplochromis burtoni: quantitative behavioral observations. Anim. Behav. 25, 964-975.
14. **Maruska, K.P. and R.D. Fernald.** (2010). Behavioral and physiological plasticity: rapid changes during social ascent in an African cichlid fish. Horm. Behav. 58: 230-240
15. **Fernald, R. D.** (2009). Social regulation of reproduction: what changes and why? Hormones, Brain, and Behavior 1: 683‑691.

16. **Fernald, R. D. and Hirata, N. R.** (1979). The Ontogeny of Social Behavior and Body Coloration in the African Cichlid Fish *Haplochromis burtoni.* Zeitschrift sür Tierpsychologie 50: 180-187.
17. **Robison, R. R., Fernald, R. D. and Stacey, N. E.** (1998). The olfactory system of a cichlid fish responds to steroidal compounds. J. Fish Biol. 53, 226-229.
18. **Cole, T. and Stacey, N. E.** (2006). Olfactory responses to steroids in an African mouth-brooding cichlid, Haplochromis burtoni (Gunther). J. Fish Biol. 68, 661-680
19. **van den Hurk, H. R. and Lambert, J. G.** (1983). Ovarian steroid glucuronides function as sex pheromones for male zebrafish,*Brachydanio rerio*. Canadian Journal of Zoology 61 (11): 2381-2387.
20. **Liberles, S. D.** (2014). Mammalian Pheromones. Annual Review of Physiology 76: 151-175.
21. **Goodson, J. L.** (2005). The Vertebrate Social Behavior Network: Evolutionary Themes and Variations. Hormones and Behavior 48 (1): 11-22.
22. **Davis, R.E., Chase R., Morris, J., and Kaufman, B**. (1981). Telencephalon of the Teleost Macropodus: Experimental Localization of Secondary Olfactory Areas and of Components of the Lateral Forebrain Bundle. Behavioral and Neural Biology 33, 257-279.
23. **Grone, B. P. and Maruska, K. P.** (2015). Divergent evolution of two corticotropin-releasing hormone (CRH) genes in teleost fishes. Frontiers in Neuroscience 9: 365.
24. **Murakami, S. L., Cunningham, L.L., Werner, L.A., Bauer, E., Pujol, R., Raible, D.W., Rubel, E.W.** (2003). Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (*Danio rerio*). Hearing Research 186 (1-2): 47-56.
25. **Stacey, N. E., Chojnacki, A., Narayanan, A., Cole, T., and Murphy, C.** (2003). Hormonally derived sex pheromones in fish: exogenous cues and signals from gonad to brain. Canadian Journal of Physiology and Pharmacology 81 (4): 329-341.
26. **Maruska, K.P., and Fernald, R.D.** (2011). Plasticity of the Reproductive Axis Caused by Social Status Change in an African Cichlid Fish: II. Testicular Gene Expression and Spermatogenesis. Endocrinology 152 (1): 291-302

27. **Kustan, J.M., Maruska, K.P., and Fernald, R.D.** (2011). Subordinate male cichlids retain reproductive competence during social suppression. Proceedings of the Royal Society B 279: 434-443.

28. **Hoshino, O., Kashimori, Y., Kambara, T.** (1998). An olfactory recognition model based on spatio-temporal encoding of odor quality in the olfactory bulb. Biological Cybernetics 79: 109-120.

29. **Sih, A, Bell A, Johnson, J.C.** (2004). Behavioral syndromes: an ecological and evolutionary overview. Trends in Ecology & Evolution 19: 372-378.

30. **Schürch, R. and Heg, D.** (2010). Life history and behavioral type in the highly social cichlid *Neolamprologus pulcher*. Behavioral Ecology 21 (3): 588-598.

31. **Riebli, T., Taborsky, M., Chervet, N., Apolloni, N., Zürcher, Y., Heg, D.** (2012). Behavioural type, status and social context affect behaviour and resource allocation in cooperatively breeding cichlids. Animal Behaviour –1-12.

32. **Heg, D.**, **Schürch, R, Rothenburger, S.** (2011). Behavioral type and growth rate in a cichlid fish. Behavioral Ecology 22 (6): 1227-1233.

33. **Hara, T.J.** (1975). Olfaction in fish. Progress in Neurobiology. 5 (4): 271-335.

34. **Westerman, R.J. and von Baumgarten, R.** (1964). Regeneration of olfactory paths in carp (Cyprinus carpio L.). Experientia. 20 (9): 519-520.

35. **Chong, K., Ying, T.S., Foo, J., Jin, L.T., Chong, A.** (2005). Aquaculture. 249 (1-4): 469-476.

36. **Rinke, E. and Wullimann, M. F.** (2004). Connections of the ventral telencephalon (subpallium) in the zebrafish (*Danio rerio*). Brain Research 1011 (2): 206-220.