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Neural Mechanisms of Sexual Transformation in *Thalassoma bifasciatum*, the bluehead wrasse

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ABSTRACT

Thalassoma bifasciatum, the bluehead wrasse, is a protogynous sequential hermaphrodite and a commonly used model for studying environmental sex determination and adult sexual plasticity. Terminal phase (TP) male bluehead wrasses demonstrate characteristic behaviors and coloration changes when courting the initial phase (IP) females in their population. Upon removal of the TP male, the most dominant IP female will begin to display these male-typical courtship behaviors and coloration changes in a remarkably short amount of time. Despite a vast depth of information surrounding the hormonal changes that occur during behavioral and gonadal transformation, there is a lack of understanding regarding the neural structures that underlie these capabilities. We have used immunohistochemistry experiments to identify the expression of early immediate genes and determined that the interpeduncular nucleus, ventrolateral thalamic nucleus, and the red nucleus are all less active in the brains of IP females performing male-typical courtship behaviors than non-courting IP females. This suggests a decline in inhibitory neural regulation is necessary for IP females to behave as courting males. Additionally, we have identified crucial brain regions behind the onset of courtship coloration by eliciting such changes in immobilized fish through glutamate stimulation of the lateral region of the ventral telencephalon, the dorsal and ventral regions of the ventral telencephalon, the supracommissural ventral telencephalon, and the dorsal habenula

INTRODUCTION

Researchers from multiple scientific disciplines have extensively studied the effects of the environment on the more abstract features of an organism, such as social behaviors and mating rituals, for generations. More recent studies have even shown how the environment can alter more measurable features of animals, such as gene expression, but the role of the environment in development is less well understood (Gilbert, 2005). Specifically, there is a lack of focus on how the environment can influence sex determination in animals. Typically, the gonadal sex of an organism is controlled by consistent and predictable patterns of chromosomal inheritance. Furthermore, this chromosomal inheritance provides instructions for the sexualization of the brain by gonadally-controlled hormone exposure, resulting in gender identity and sex-specific attributes and behaviors (Cooke et al., 1998). However, this standard model does not hold true for all animals- especially those found below the surface of our oceans.

In approximately three percent of fish, gonadal development as well as neural aspects of sex determination and related behaviors are contingent upon environmental factors, such as population density, resource availability, or temperature (Price et al., 2018; Jobling, 2002; Warner and Swearer, 1991; Devlin and Nagahama, 2002). Remarkably, gonadal development and sex-specific behaviors remain plastic in some fish, meaning they maintain the ability to alter their sex even as adults (Godwin, 2009). One such species is the bluehead wrasse, *Thalassoma bifasciatum* (Warner and Swearer, 1991).

The bluehead wrasse is a protogynous sequential hermaphrodite, meaning individuals can change only from female to male, that exists in two coloration phenotypes: yellow Initial Phase (IP) males and females and dichromatic Terminal Phase (TP) males that display the characteristic blue head (Foran and Bass, 1999) (Figure 1). All bluehead wrasse begin as either a male or female IP and some later develop into a TP male, either by maturation of an IP male or sexual and behavioral transformation of an IP female (Warner and Swearer, 1991). The initial sex determination of the IP fish is a function of population density. Small schools with less than 100 individuals contain a small percentage of males while much larger schools may contain up to 46% IP males (Warner and Schultz, 1992; Munday et al., 2006). In each school a single, territorial TP courts a large population of IP females with elaborate displays of male-specific mating behavior such as circling vibrations above the female, inspections of the female abdomen, and mock spawn rushes (Warner, 1975; Perry and Grober, 2003).

In a laboratory environment, these courtship behaviors can be monitored in selectively curated populations, allowing observations of the transformation of IP females into TP males through TP removal studies (Price et al., 2018). In these studies, as in natural populations, when the resident TP male is removed from a population (via capture or predation), the most dominant IP female transforms both behaviorally and sexually into the reef's new TP male. The sexual transformation requires a minimum of 8-10 days to manifest and is dependent on the upregulation of androgens (Semsar and Godwin, 2003). The behavioral transformation, however, is far more rapid. Competent IP females can begin displaying male-typical courtship behavior within 24 hours of TP male removal (Warner and Robertson, 1975; Warner and Swearer, 1991). Consistent with its rapid

onset, behavioral transformation has been noted to occur independently of gonadal androgens, thereby indicating that it is under neural control. Furthermore, the change in behavior is correlated to increased levels of hypothalamic arginine vasotocin (AVT), the non-mammalian homolog of arginine vasopressin (Semsar and Godwin, 2003).

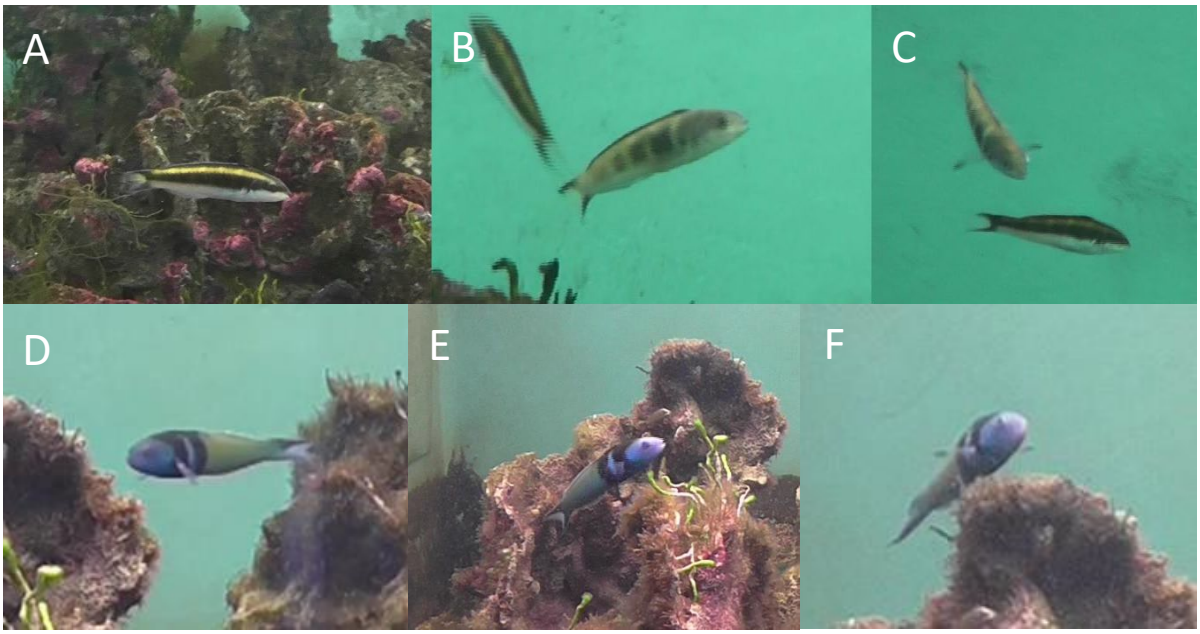


Figure 1: Non-courting and Courting Coloration Changes of IP females and TP males
A) Non-courting yellow IP female **B)** IP female displaying male-typical courtship coloration with opalescent head, lighter body, and lighter opercular patches **C)** Same courting IP female as (B) showing darkened pectoral fin tips **D)** Non-courting TP male with characteristic blue head **E)** Courting TP male displaying opalescence **F)** Same courting TP male as (E) showing darkened pectoral fin tips

Within a particular reef population, IP females interact aggressively to establish a social dominance hierarchy. The order of individuals within the hierarchy is usually correlated closely to relative size (Warner and Swearer, 1991; Warner and Schultz, 1992). This hierarchy is particularly evident after TP removal as it is usually the most dominant, largest IP female that first begins to display male-typical courtship behaviors and begin gonadal transformation (Warner and Swearer, 1991; Price et al., 2018); lower-ranking IP females show much longer latencies for behavioral transformation. This

decreased latency to transform among more dominant IP females has led to the development of the 'priming' hypothesis which posits that IP females become more competent to transform as they ascend the social hierarchy (Price et. al., 2018). Such a model challenges the previously accepted notion that the physiological forces that drive behavioral transformation and gender plasticity occur rapidly and begin only when the opportunity to transform arises. Instead, this model suggests that subtle changes improving the readiness of an IP female to transform, such as the upregulation of AVT receptors, occur gradually over a longer period of time as an individual ascends the social dominance hierarchy (Price et, al., 2018).

Previous studies have increased our understanding of the endocrine changes that accompany these sexual and behavioral transformations. The bluehead wrasse, as with several other protogynous species, display increased levels of 11-Ketotestosterone (11-KT), an endogenous androgenic sex hormone and decreased levels of 17β Estradiol (E_2) and aromatase during transformation (Semsar and Godwin, 2003; Lamm et. al., 2015; Marsh-Hunkin et al., 2013). Furthermore, increased AVT receptors (AVTRs) via upregulation of the V_{1a2} gene are correlated with the onset of male-typical courtship behaviors in IP females (Semsar and Godwin, 2003; Lema et al., 2012). This neurohormone is produced in the posterior pituitary and is well conserved among vertebrates with respect to its involvement in the regulation of social and sexual behaviors, such as courtship and aggression (Godwin, 2010). Another neurohormone, gonadotropin releasing hormone (GnRH), is also of great importance for inducing sex change and eliciting territorial and aggressive male-typical behavior (Grober, 1991).

Understanding the role of these neurohormones in the process of sexual transformation can help identify specific brain regions that may vary between sexual phenotypes and be involved in the priming process. For instance, AVTR mRNA is more abundant in the magnocellular region of the pre-optic area of the hypothalamus (mPOA) during the sexual transformation (Semsar and Godwin, 2003), and TP males have been reported to have larger and more abundant GnRH releasing neurons in the POA (Lamm et al., 2015). Functional areas in this brain region are likely to be implicated in the control network behind behavioral transformation.

Despite the depth of understanding surrounding the neurohormone contributions to sexual and behavioral transformation in the bluehead wrasse, there is a significant gap in knowledge of the correlated neural structures and circuits that govern male-typical courtship behavior and are the targets of neuroendocrine regulation. Functional analysis of forebrain areas hypothesized to be involved, such as those in the POA, and studies that determine brain regions that are differentially active between IP females that are or notdisplaying male-typical courtship activities are needed to reveal these unresolved structures and networks and the nature of their regulation over sex and behavioral transformation. This research aimed to meet these needs by employing immunohistochemical investigation of immediate early gene expression and targeted glutamate iontophoresis in the wrasse brain.

MATERIALS AND METHODS

Animal Care

IP fish were caught from the reefs of Key Largo in the Florida Keys by Caribbean Tropicals Inc. and shipped overnight to Salt Lake City, usually arriving within 72 hours of capture. Upon being received, the fish were examined to determine sex. This involved an initial anesthetization in a 0.05% solution of MS-222 in seawater followed by maintained sedation with a more dilute 0.01% solution. Sexing was performed by examining the external genital structures of the fish with the aid of a microscope. If a more prominent papilla could be identified, the IP fish is male, while a smaller papilla and larger genital pore are indicative of IP females (Warner and Swearer, 1991). At this time, the length of all fish was also measured and recorded. This information from sexing and sizing was used to create populations of five to seven IP females with a size difference of at least 4mm between each female to distinguish individuals and create a social hierarchy. The ranking of IP females in the social hierarchy descended from largest to smallest, with the titles first-order, second-order, and so on appointed by relative length (largest being the first-order). All IP female populations were housed with at least one TP male to prevent premature sex or phase change.

Experimental populations, always consisting of only IP females and a TP male, were placed into one of two 800-gallon aquaria (1.2x1.8 m floor area, 1.2 m deep) in a temperature-controlled greenhouse environment. Fish were exposed to the natural light:dark cycle for Salt Lake City, Utah unless natural sunlight failed to last for 12 hours, wherein artificial light was added in supplement. Temperature of the water in the tanks was measured daily and maintained within 2 degrees of 24˚C at all times of year. Sea water was made by dissolving Instant Ocean Sea Salt (Spectrum Brands, Blacksburg, VA) in deionized water. Specific measurements of water quality and solute levels (pH,

calcium, nitrates/nitrites, ammonia, and phosphates) were taken bi-annually via Reef Lab aquarium test kits (Red Sea, Houston, TX). If measurements were outside the norm for a naturally environment, adjustments to the water content were made to rectify the imbalance.

Fish were fed daily from a diet of frozen mysis shrimp (Hikari Bio-Pure, Wayward, CA) and/or live brine shrimp collected from the Great Salt Lake, except on the days before a planned removal. On these days, food was withheld to increase likelihood of capture the next morning using bait. Food was added to the tanks following the activation of recirculating pumps. Fish were conditioned to receive food, after pump activation, from a triangular outlet built into the aquaria with a sliding glass sheath that could be released by an observer at any time. When released, this sheath separated the fish from the rest of the tank and confined it to the triangular outlet, from which they were removed by net. This conditioning of the fish to swim under the glass sheath to feed everyday made capture relatively easy when necessary.

Behavioral Observations

Each 800-gallon aquarium was fitted with glass viewing windows (0.61m x 0.61m) to allow observation and video recording of the populations inside. To simulate a semi-transparent mirror, thereby concealing the observer from the fish, the observation areas on the outside of these windows were surrounded by dark booths constructed from black plastic and PVC pipes. Fish were monitored and scored for dominance and courtship behaviors. Notable behaviors were based on previous protocols published in Price et. al. (2018) and characteristic movements and interactions observed in field studies (Warner and Swearer, 1991; Dawkins and Guilford, 1993). Scores were recorded

for the following behaviors: aggression, darkening of pectoral fin tips, emergence of a white saddle just rostral of the head, opalescent coloration, inspections, glides, vibrations, pectoral fin fluttering, circling, mock spawn rushes, and paired spawn rushes. Scores were recorded in person or from records of video footage that was taken every day from sunrise to sunset from each tank.

All animal experiments conducted in this study were approved by policies 14-07009 and 17-06009 of the University of Utah's Institutional Animal Care and Use Committee.

Immunohistochemistry

There were three main groups of fish analyzed in the immunohistochemistry experiments. These were primed courting fish, primed non-courting fish, and unprimed non-courting fish. With the aid of the dominance hierarchy, built-in trap mechanism, and 24-hour behavioral observations, we were able to capture fish with confidence regarding their priming status. Priming status refers to how competent an IP female is to display male-typical courtship behaviors (Price et. al., 2018). Capture of primed courting fish took place after a significant display of courtship activity, wherein opalescence, black fin tips, vibrations, and mock spawns were observed. These bouts of courtship lasted anywhere from 30 to 60 minutes, and an additional waiting period of 45-60 minutes was allotted following the courtship to allow enough time for mRNA products of relevant immediate early genes (IEGs) to be upregulated. Capture of primed, non-courting fish took place after careful observation lasting at least 4 hours to ensure that no male-typical courtship behaviors were displayed (although primed fish must have previously

performed these behaviors to be labeled as such) and therefore IEG product levels remain at baseline.

Post capture, the fish were removed from the trap, measured, and anesthetized in a 0.05% MS-222 in seawater solution. They were then respirated with a 0.01% MS-2222 in seawater solution and perfused with 15ml of Hinkman ringer (0.0067% heparin, 0.017% MS-222) and 25ml of a recently prepared 4% solution of paraformaldehyde (PFA) to fix the desired tissues. Following perfusion, the brain and gonadal tissues are removed and stored in a 4% PFA solution overnight at 4˚C. Tissues were then cryoprotected via a series of sucrose solutions (an hour soak in 5%, 10%, and 20% followed by an overnight soak in 30%) and stored at -20˚C. For all tissue samples used in this study, fish were captured between 2016 and 2018 and no tissues were kept in storage for more than a year.

When ready for histology, brains were removed from storage and mounted in Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA) and transferred to a -20˚C Microm HM550 Cryostat (MICROM International GmbH, Walldorf, Germany) where they were transversely sliced into 50µm sections and placed into wells. Brain sections were then washed four times with phosphate buffered saline (PBS) followed by an hour treatment with blocking solution (2% bovine serum albumin (BSA) and 0.03% Triton-X in PBS) while oscillating on a rotating shaker. Sections were then treated with the primary antibody solution, either a 1:250 mixture of polyclonal rabbit anti-c-Fos IgG (100µg antibodies/ml PBS; Santa Cruz Biotechnology Inc, Dallas, TX) or 1:30,000 polyclonal rabbit anti-Ps6 IgG (11.8 mg antibodies/ml PBS; Sigma-Aldrich, St. Louis, MO) in carrier solution (2% BSA, 0.01% Triton-X, PBS) overnight at

4˚C on a rotating shaker. Finally, all sections were treated with the same secondary antibody solution, a 1:500 mixture of goat anti-rabbit IgG conjugated with Cy3 for fluorescence (Jackson ImmunoResearch Laboratories, West Grove, PA) in carrier solution overnight at 4˚C on a rotating shaker. Slices were then mounted with Fluoromount-G (Southern Biotech, Birmingham, AL) and stored at 4˚C away from light.

Mounted brain sections were imaged with an Olympus BX50 fluorescent microscope and camera system (Olympus Corporation, Center Valley, PA). Sections were imaged in pieces to obtain higher resolution and then merged together to form one image per section using Photoshop Lightroom version 2015.8 (Adobe, San Jose, CA). Fluorescent intensity was measured using ImageJ version 1.50i. Variance in pixel intensity resulting from unequal exposure and antibody concentrations was adjusted and normalized by calculating a ratio of signal intensity over background (the average fluorescence of the entire section) for each section being measured, thereby only quantifying the degree to which any given area was more illuminated than the surrounding tissue. The c-Fos and Ps6 markers used in the histology are proxies for neuronal activity. Therefore, wherever expression of either of these IEGs is found, via detection of illuminated pixels, it can be determined that there was significant neuronal activity prior to capture.

All statistical analyses for the immunohistochemistry experiments were conducted using R version 3.5.2. A two-tailed, Mann Whitney U test with $\alpha=0.0167$ was used to compare measurements for neural activity between primed courting, primed non-courting, and unprimed non-courting fish. Graphs were composed on SigmaPlot version 11.0 (Systat Software Inc., Chicago, IL).

Glutamate Stimulation

To conduct glutamate iontophoresis experiments, we fitted a small plexiglass tank with seawater kept at 25˚C with a body clamp and glass tube respirator. Fish used in these experiments were caught from the 800-gallon experimental aquaria or small holding tanks and lightly anesthetized with 0.05% MS-222 in seawater. Muscle activity was inhibited by intramuscular injection of pancuronium bromide (8mg/ml, 2µl/g fish). Fish were then suspended in the tank by the clamp and respirator tube, and the water level adjusted such that the dorsal surface of the head remained dry. Lidocaine hydrochloride jelly (2%, Akorn Inc., Lake Forest, IL) was applied to the cranial ectoderm to numb before a small hole was made in the cranium using a Foreman series AB dental drill (Foredom Electric Co., Bethel, CT) to expose the brain. Glass electrodes (A-M Systems, Sequim, WA) were pulled on site as needed with a Sutter Model P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA) and filled with 0.1M glutamate (pH=8.0) for excitation or 20mM biocytin for cell labeling.

Glutamate was iontophoresed into the brain (-100nA for approximately 10 seconds per trial) using a Duo 773 Electrometer (World Precision Instruments, Sarasota, FL) and effects on the fish melanophores were observed through projection of a Dino-lite AM312T high-resolution live video recorder (Dunwell Tech, Torrance, CA) to a computer monitor. The forebrain was probed to identify stimulation sites that elicited melanophore/chromophore changes consistent with those observed during male-typical courtship behaviors (darkened fin tips, opalescence, emergence of a white saddle). Furthermore, when relevant coloration changes were induced, the amplitude and duration

of iontophoretic current were varied to determine the thresholds for the evoked responses. This information, coupled with our knowledge of the fish's sex and priming status, could provide helpful insight as to what brain regions differ in function between TP males, IP males, and primed and unprimed IP females. Regions shown to control courtship coloration were marked with biocytin which was iontophoresed at +100nA for approximately 45 seconds.

Precise control of the electrode's position was managed in two ways. Movement along the x and y (horizontal) planes of the electrode holder was accomplished via manual adjustments guided under a dissection microscope with an eyepiece reticule. Movement along the z (vertical) plane was controlled by a Burleigh 6000 Controller (Burleigh Instruments Inc., Fisher, NY). Video (recorded at 1280x1024 pixel resolution and 15 fps) and audio files from all stimulations were recorded and integrated with corresponding data from the Duo 773 Electrometer and a keyboard in Spike 2 version 7.0 (Cambridge Electronic Design Limited, Milton, Cambridge, England). For data processing, the video files were converted into still images using Free Video to JPG Converter version 5.0.101 and coloration changes were quantified using ImageJ version 1.50i. Quantified data from ImageJ was analyzed using R version 3.5.2 and RStudo version 1.1.463.

At the close of each experiment, fish were perfused with Hickman ringer (0.0067% heparin, 0.017% MS-222) and a 2.5% solution of glutaraldehyde. Brains were removed and stored in 2.5% glutaraldehyde at 4˚C. For all fish used in this study, duration of the experiment did not exceed one day, and no brains were kept in storage for longer than one year.

Brains were then sliced into 100µm sections by a Vibratome Series 1000 Sectioning System (Technical Products International, Inc., St. Louis, MO). Biocytin was visualized using a Vestastain Elite ABC kit (Vector Labs, Burlingame, CA), counterstained with a 0.05% solution of Neutral Red, and dehydrated in a series of ethanol solutions (less than one minute each in 50%, 70%, 95%, 100%) and AmeriClear. Finally, the processed brain sections were mounted using permount.

RESULTS

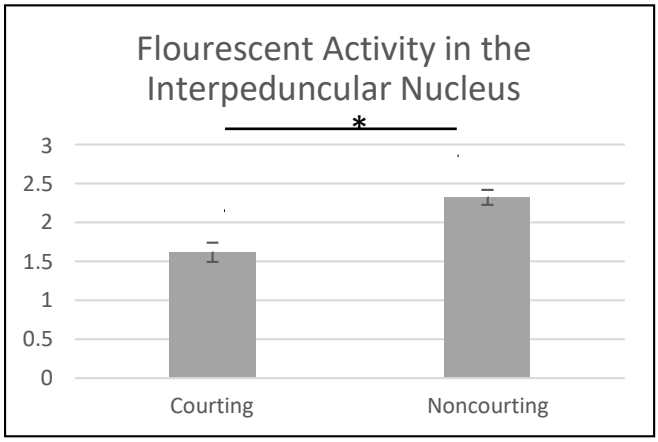
Immunohistochemistry

Brains (n=21) treated with the c-Fos antibody were taken from fish in 4 treatment groups: courting (n=6), non-courting (n=9), unprimed (n=5), and 1 sensory deprived fish that was held in a dark, soundproof room for 24 hours before perfusion. Data from these samples were analyzed in conjunction with the Ps6 treated brains (n=18) from courting (n=7), non-courting (n=7), unprimed (n=3), and 1 fear conditioned fish that had narrowly escaped the trap mechanism at first and was subsequently captured after displaying signs of stress.

From these samples, we were able to identify three regions with significantly different levels of activity between courting and non-courting fish. The interpeduncular nucleus (IPN) showed greater labeling for c-Fos (n=13) and Ps6 (n=10) in the brains of non-courting than courting wrasses (Mann-Whitney U-Value=10, $p<0.00001$) (Figure 2).

Labeling for c-Fos (n=19) and Ps6 (n=9) in the ventrolateral thalamic nucleus (VL) was also significantly greater in the brains of non-courting vs. courting fish (Mann-Whitney U-Value=24, p=0.00078) (Figure 3).

Analysis of Ps6 labeling in a region close to the IPN, the Red Nucleus, from courting (n=5) and non-courting (n=5) revealed significantly greater activity in the brains of non-courting than courting wrasses (Mann-Whitney U-value=0, p=0.01208)(Figure 4).



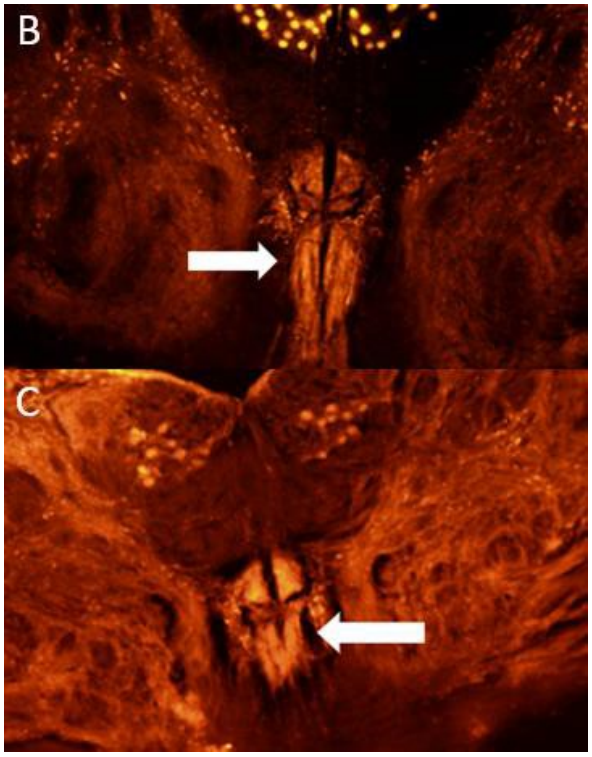
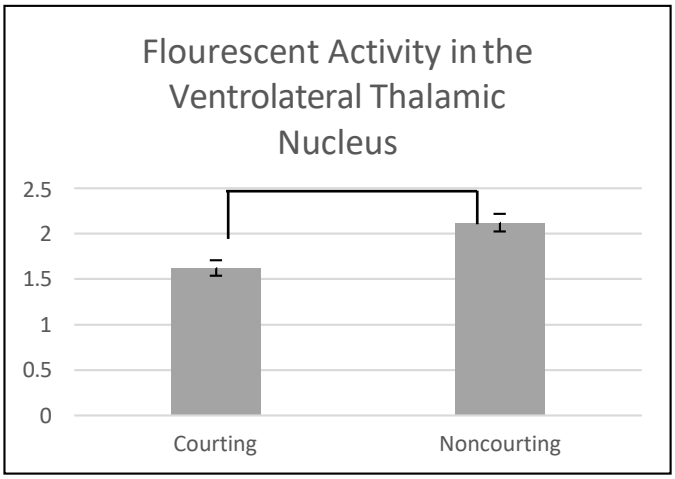


Figure 2: Immunohistochemistry Results in the Interpeduncular Nucleus
A) Relative activity of the IPN in courting and non-courting fish measured by degree of fluorescent activity. Asterisk represents a statistically significant relationship.
B) Image of a cross section of a non-courting brain expressing c-Fos. Arrow indicates the Interpeduncular Nucleus
C) Image of a cross section of a courting brain expressing c-Fos. Arrow indicates the Interpeduncular Nucleus



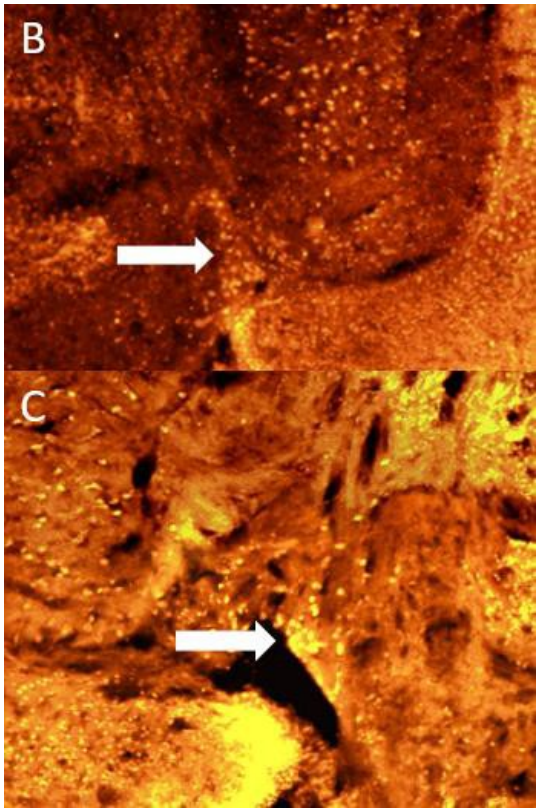
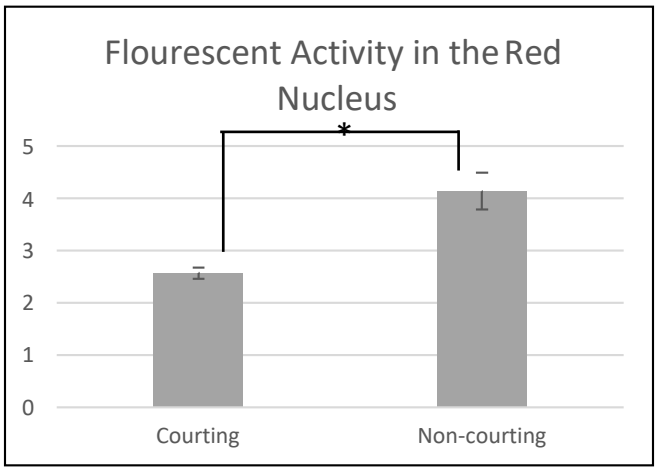


Figure 3: Immunohistochemistry Results in the Ventrolateral Thalamic Nucleus (VL)
A) Relative activity of the VL in courting and non-courting fish measured by degree of fluorescent activity. Asterisk represents a statistically significant relationship.
B) Image of a cross section of a non-courting brain expressing c-Fos. Arrow indicates the VL.
C) Image of a cross section of a courting brain expressing c-Fos. Arrow indicates the VL.



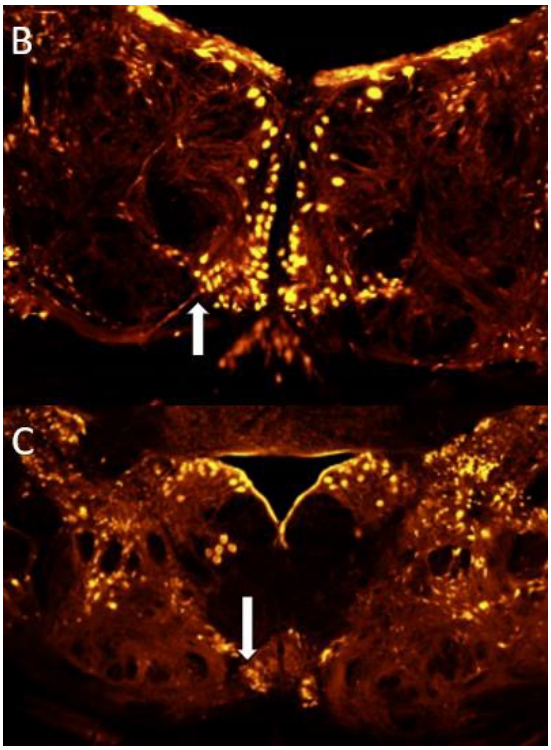


Figure 4: Immunohistochemistry Results in the Red Nucleus
A) Relative activity of the Red Nucleus in courting and non-courting fish measured by degree of fluorescent activity. Asterisk represents a statistically significant relationship.
B) Image of a cross section of a non-courting brain expressing Ps6. Arrow indicates the Red Nucleus.
C) Image of a cross section of a courting brain expressing Ps6. Arrow indicates the Red Nucleus.

Glutamate Stimulation

Using the regions identified in the immunohistochemical analyses as a guide, we conducted 23 glutamate iontophoresis experiments. From these, we were able to elicit eight unique effects that often occurred in conjunction with one another: pectoral fin darkening; opercular patch lightening and darkening; body lightening and darkening; induction of blue pigment in the head; and horizontal band formation (Figure 5).

Identification of the corresponding biocytin marking in the treated brain slices indicate three regions where an identical response had been observed on at least two other fish. These regions are the lateral region of the ventral telencephalon (Vl) which caused patch lightening when stimulated and the dorsal and ventral regions of the ventral telencephalon (Vd and Vv) which both caused darkening of the body, head, and patches when stimulated (Table 1).

Although we could not find one brain region to be responsible for induction of a blue head in three or more experiments, we were able to cause this effect on two occasions with corresponding markings indicating the supracommissural ventral telencephalon (Vs) in one and the dorsal habenula (dHb) in the other (Figure 5d). This effect is particularly exciting as it shows a clear relationship to sexual transformation as only terminal phase males have a blue head (Table 1).

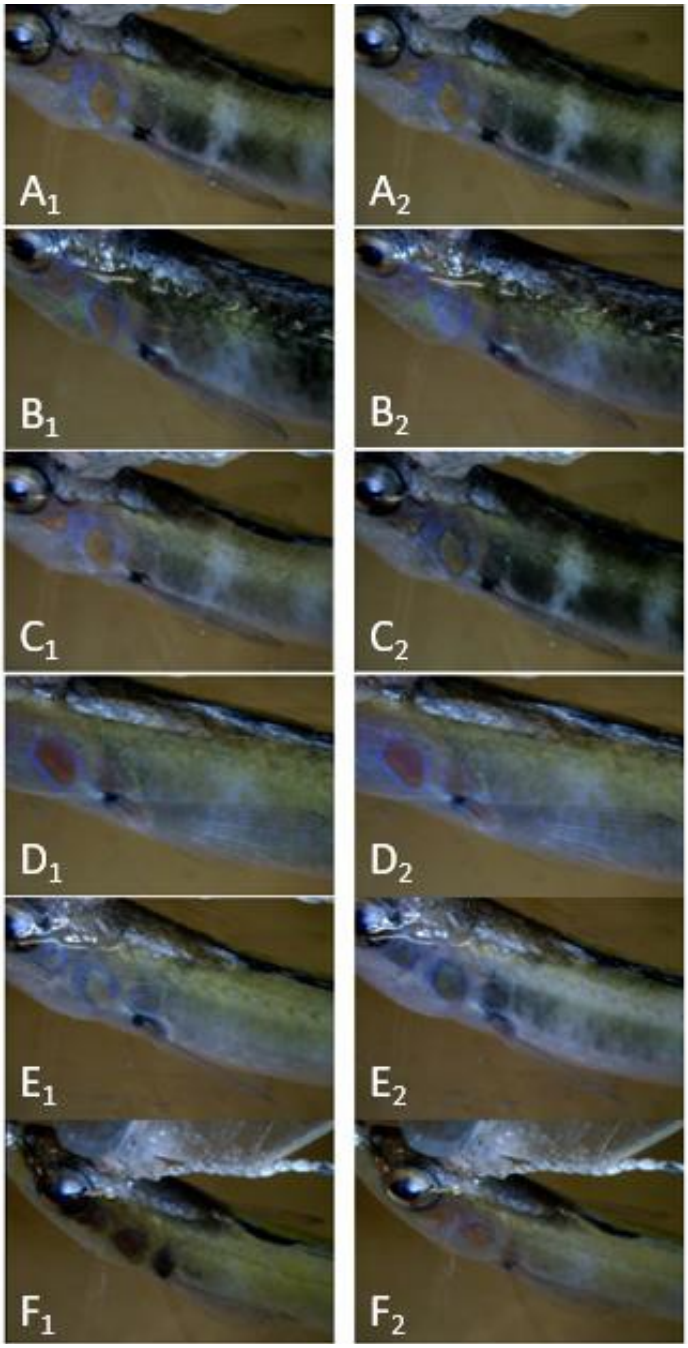


Figure 5: Changes in IP female patterning and coloration resulting from glutamate stimulation

A) Darkening of pectoral fins. **B)** Patch lightening. **C)** Head, body, and patch darkening. **D)** Induction of blue pigment in the head. **E)** Formation of a dark, horizontal band across the body. **F)** Head and patch lightening.

Brain Region Stimulated	Elicited Color Change
Lateral Region of the Ventral Telencephalon (Vl)	Opercular Patch Lightening
Dorsal Region of the Ventral Telencephalon (Vd)	Body, Head, and Opercular Patch Darkening
Ventral Region of the Ventral Telencephalon (Vv)	Body, Head, and Opercular Patch Darkening
Supracommissural Ventral Telencephalon (Vs)	Blue Head Induction
Dorsal Habenula (dHb)	Blue Head Induction

Table 1: Compiled Results of Glutamate Stimulation Experiments

There are two distinct regions in the head area that we monitor for pigment change during the glutamate iontophoresis. These are the opercular patches, pink hued circular areas just caudal of the eye, and the annulus, a blue ring that encircles the opercular patch (Figure 6). Change in opercular patch coloration is important during male-typical courtship behaviors as these areas lighten when the fish becomes opalescent. The annulus, which becomes more distinctly blue during IP courtship, is of primary interest as the relative levels of blue pigmentation, comparable to the head pigmentation of TP males, are a useful tool to compare likely priming status in IP females.

Visual observations throughout the glutamate iontophoresis experiments were insufficient to determine if the opercular patches and annulus always exhibited the same response to a relevant stimulation. A goal for further analysis was to test whether stimulation of discrete regions of the forebrain elicit coloration changes (lightening or darkening) in specifically the opercular patch or annulus, or both; that is, do particular regions control specific vs multiple changes in coloration of specific or multiple peripheral sites? Based on previously described models of chromatophore innervation in

teleosts, where severance of one efferent nerve led to a uniform change in pigmentation throughout an entire somatic region (Pye, 1964; Parker, 1934; and Abramowitz, 1936); we felt it unlikely that these concentric features would be controlled independently and expected any measured lightening or darkening in the opercular patch to be mirrored by the annulus and vice-versa.

To test this hypothesis, we generated plots of annulus and opercular patch responses to glutamate stimulation in 35 different penetrations across 11 different fish wherever a significant change in opercular patch and/or annulus coloration was noted. We found one incidence of an uncoupling of stimulation effects for the two regions and two other cases where the amplitude of stimulation-induced changes differed appreciably for these regions elicited (Figure 7). To complete this analysis, we ran a binomial distribution test and were able to conclude that the opercular patch and annulus generally respond together and appear to not be under independent neural control ($p < 0.0001$).

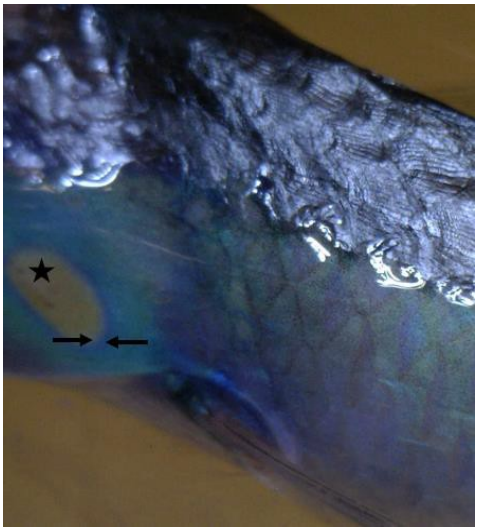


Figure 6: Patches and Annulus on a TP male. Image captured from the 43rd penetration on a TP male during a Nov. 29, 2018 experiment. A black star shows the patch and black arrows indicate the annulus.

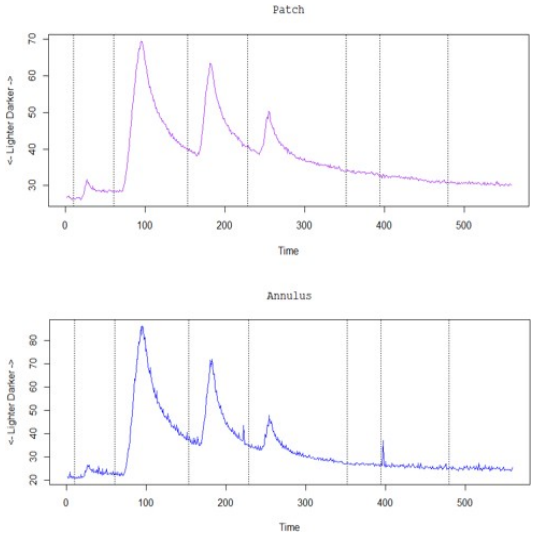


Figure 7: Single incident of differential response to stimulus in Patch and Annulus.
Plots generated from the 25th penetration of an experiment on Sept. 14, 2018. Vertical lines indicate glutamate release. The blue trace was obtained from the annulus, purple from the patch. Differential response observed at time 400.

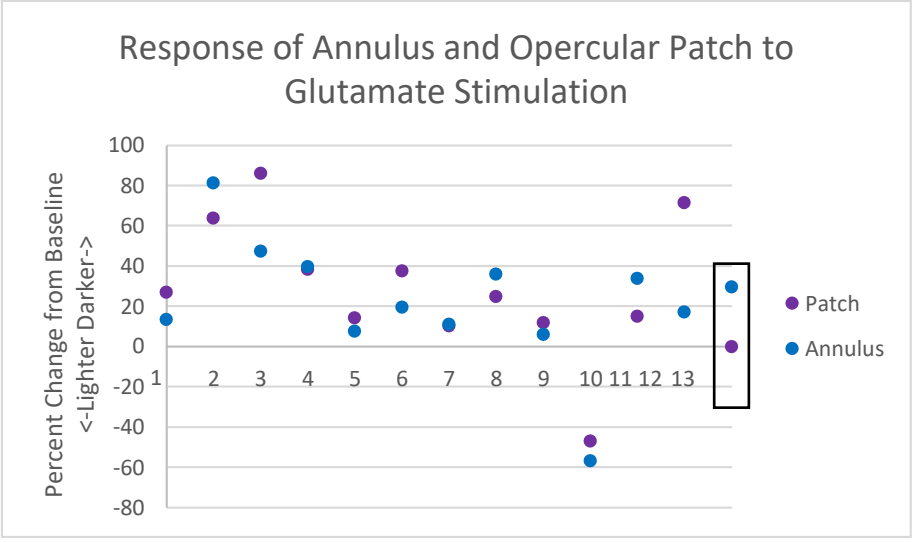


Figure 8: Summary of Opercular Patch and Annulus Responses to Stimulation
Data from 13 glutamate stimulations were selected to compare the type of response (lightening or darkening) as well as degree of response for the patch and annulus. The single incident of differential response to stimulus presented in Figure 7 is shown here at stimulation 13 (black box).

While the overall response of the patches and annulus to stimulation is highly similar, these areas often differ in their degree of response (Figure 8). One potential explanation for this difference in response strength is that these regions, while not under

completely independent innervation, may be controlled by close but distinct subnuclei and therefore do not show an exact mirroring of chromophore response following neural excitation.

DISCUSSION

Ventrolateral Thalamic Nucleus

The ventrolateral thalamic nucleus, VL, was shown to be less active in courting fish than non-courting fish in the immunohistochemical data (Figure 3). This nucleus is among three prethalamic structures (VL, VM, and I) that have no known mammalian homolog (Mueller, 2012; Mueller and Guo, 2009). This lack of reference to mammalian structures makes understanding the exact role of this nucleus in teleosts quite difficult. However, hodological and immunohistochemical studies have shown the VL to be a GABAergic nucleus involved in the visual system that projects to the preglomerular complex (Mueller, 2012; Mueller and Guo, 2009; Yamamoto and Ito, 2008). The preglomerular complex in teleosts has functional homology to the sensory integration and relay activities of the mammalian thalamus (Yamamoto and Ito, 2008).

Due to the inhibitory nature of this visual nucleus, it is possible that decreased activity levels allow certain behaviors that are otherwise inhibited. This is consistent with the measured immediate early gene expression in the VL being higher in non-courting than courting fish. Inspections are one of the earliest signs of male-typical courtship and involve the male investigating how gravid the female is by way of swimming beneath her and looking up at her underbelly (Warner, 1975; Perry and Grober, 2003). Perhaps the

visual stimulus of a gravid female ready for courtship leads to decreased activity in the VL which allows for the onset of higher-order courtship behaviors.

Stimulation of this region during glutamate iontophoresis experiments has been shown to induce fin tip darkening on one occasion. Further experiments are needed to confirm if this is a reliable response that would further indicate the importance of the VL in regulating male-typical courtship morphology and behavior.

Red Nucleus

The red nucleus, or nucleus ruber, identified by immunohistochemistry experiments is known to be important for forelimb control in mammals (Carpenter, 1956; Ghez & Kubota, 1977; Lawrence & Kuypers 1968), and has been linked to indirect innervation of pectoral fin motor neurons in other teleosts (Nakayama et al., 2019; Nakayama et al., 2016; and Matsui, 2017). It is likely that this nucleus plays an important role in regulating normal (non-courtship-relating) swimming through this indirect pectoral fin innervation. When the activity of the red nucleus is decreased, as was seen to be the case in courting fish, abnormal movements of the pectoral fins are possible. This is not trivial as quick movement of the pectoral fins is required for pectoral fin fluttering and suspended pectoral fin movement is required for gliding, both characteristic male-typical courtship displays (Dawkins and Guilford, 1993; Warner and Schultz, 1992).

Interpeduncular Nucleus

The IPN is often described in junction with the medial habenula (MHb) as a part of the highly conserved MHb-IPN pathway which has been implicated in reward, motivation, fear and anxiety, and sleep (Xu et al., 2018; McLaughlin et al., 2017; and

Subedi, 2015). Specifically, activation of the IPN has been linked to behavioral inhibition (Wolfman et al., 2018). The lower activity in this inhibitory region in courting fish compared to non-courting fish, as indicated by immunohistochemical results, may serve to release the onset of courtship in fish by removing the normal levels of inhibition on brain regions that are active during courtship. Similar to reasonings explaining why a decrease in fear may be necessary to exhibit male-typical courtship behaviors, it is likely that this region normally functions to block behaviors that would make a wrasse more at risk for predation (Robertson and Hoffman, 1977).

Lateral Region of the Ventral Telencephalon

Stimulation of the Vl elicited lightening of the opercular patches, a change that is associated with the appearance of fish performing male-typical courtship behavior (Figure 1b). This region is thought to be homologous to the mammalian nucleus basalis, a relay center for cholinergic neurons responsible for widespread innervation of the cortex (Rink and Wullimann, 2004; Gilissen et al., 2001). The widespread projections of the Vl could explain why no significant difference in activity levels in courting vs. non-courting fish were detected in the immunohistochemistry studies despite its clear relevance to male-courtship behavior shown in the glutamate iontophoresis experiments.

One possibility is that the Vl is a functionally heterogeneous region with only a small subnuclei controlling opercular patch lightening that is not detectable by the measurements of activity taken from the entire region during immunohistochemical analysis. Furthermore, the additional functions of the Vl may allow the net activity of the entire region to remain constant, despite fluctuations in subnuclei activity levels. For example, during courtship behavior, a subset of Vl neurons may be active and elicit patch

lightening, while those that project to the amygdala homolog (Vs) may be less active (Wenk, 1997); thus, the immediate early gene levels reflects no *overall* shift in activity in the Vl region.

This decreased activation of the amygdala is consistent with the behavioral changes needed to perform male-typical courtship behaviors. Studies of natural populations of bluehead wrasses have noted an elevated number of instances of predation on spawning bluehead wrasses (Robertson and Hoffman, 1977). To minimize risks of predation, bluehead wrasses typically avoid the water column above the coral. However, courtship displays and spawning are performed in this space, thereby requiring some inhibition of a self-preserving fear response. Additionally, throughout our behavioral observations we noticed that non-courting fish were wary to stay in the trap for extended periods of time and some were hesitant to enter at all. These same fish became far less trap shy while courting which supports the proposed idea that decreases in fear-related activity are tied to increases in male-typical courtship behavior.

Induction of Blue Head Pigmentation

Our ability to induce blue pigment in the heads of IP female fish via glutamate stimulation of the Vs and dHb indicates that the capability to express this phenotype may always be present or is developed before transformation occurs, consistent with the ideas of the priming hypothesis (Price et. al., 2018). Neither of the fish that displayed this color change were in an opportune setting to transform at their time of capture. One was housed with a more dominant IP female present and the other was housed with a TP male. This provides further support the idea that the immediate opportunity to transform does not have to be present for an IP female to be capable of expressing blue head

pigmentation. However, an insufficient amount of data exists for this to be greater than guiding speculation and future studies should be conducted to investigate further.

Dorsal and Ventral Regions of the Ventral Telencephalon

The Vv has been identified to be a homolog to the mammalian lateral septum, a known component of the conserved social behavioral network (Goodson, 2005). A crucial aspect of this social behavioral network is the role it plays in reward and regulating reproductive behavior (Newman, 1999). Identifying an exact mammalian homolog for the Vd has been far more controversial, but some consider this region to be part of the teleost subpallium which is responsible for controlling behavior and administering reward (Moreno et al., 2009; Ganz et al., 2012). For these reasons, the Vd and Vv were among the first candidate regions analyzed from our immunohistochemistry data, but we found no significant difference in the level of c-Fos expression in courting vs. non-courting IP females for either nuclei. Moreover, glutamate stimulation of these regions elicited body, head, and opercular patch darkening that are more typical of non-courting IP females as oppose to the expected lightening in these regions that occurs during male-typical courtship (Figure 1).

Due to the negative results of immunohistochemical analysis and the nature of the elicited color change, we considered the possibility that the activity of these nuclei are not relevant to male-typical courtship behavior or presentation. However, this is unlikely considering the role of the Vv and Vd in behavioral networks coupled with the findings of other research that that directly links Vv activity to courtship behavior in the teleost *Oncorhynchus nerka* (Satou et. al 1984). What is more likely is a similar explanation as was proposed for the Vl. The Vd and Vv receive a vast amount of connections and may

also be functionally heterogenous which would result in the overall levels of c-Fos expression remaining relatively constant in courting and non-courting fish despite a change in situational functioning (Atoji and Wild, 2004).

Furthermore, studies of the social behavioral network across vertebrates have shown that while immediate early gene expression in any one specific nucleus may be similar across different activities, an analysis of the network as a whole reveals drastic changes in overall activity patterns (Goodson, 2005; Newman, 1999; Sakata and Crews, 2004; Sakata et al., 2000). With this consideration, new or additional methods that do not rely on measuring total activity levels at one particular locus may be beneficial in progressing our understanding of these complex nuclei and their potential relevance to courtship and social sex determination in the bluehead wrasse. Additionally, the current design of the glutamate iontophoresis experiments does not allow detection of motor responses to brain stimulation beyond the potential to induce body vibrations near the end of experiments when recovery of gilling was observed. This limitation may prevent us from identifying regions that control elements of male-typical courtship behavior outside of transient nuptial coloration.

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