5-2017

# Processing of Visual and Social Stimuli in the Green Anole Lizard Brain

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PROCESSING OF VISUAL AND SOCIAL STIMULI IN THE GREEN ANOLE LIZARD BRAIN

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A DEPARTMENT HONORS THESIS SUBMITTED TO THE

PROGRAM OF NEUROSCIENCE AND THE DEPARTMENT OF BIOLOGY AT TRINITY UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR GRADUATION WITH DEPARTMENTAL HONORS

DATE _____April 11, 2017_____

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**Abstract**

Many studies of animal behavior use video technology to mimic live interactions and minimize the variability of natural animal behavior. Here, I seek to understand how information processing differs among *Anolis carolinensis* (green anole lizards) exposed to a live lizard compared to a video representation of a lizard. I conducted behavioral trials in which I placed a male lizard in a visually neutral arena, presented it with visual information from a live anole or from carefully constructed video playback, and recorded their behavioral responses. Each lizard (n=40) was randomly assigned to one of four treatments – Live Anole (two live males interacting with each other), Anole Video (focal lizard shown video of a lizard displaying on a perch), Scrambled Video (focal lizard shown video of a lizard displaying on a perch, but with the pixels scrambled to remove social context), or Control (focal lizard shown video of a stationary perch). Immediately after each trial, lizard brains were flash-frozen in isopentane. To measure neural activity, I then used immunocytochemistry to quantify expression levels of the immediate early gene c-*fos* in two visual brain regions, the Nucleus Rotundus (NROT) and Lateral Geniculate Nucleus (LGN), and one social brain region, the Pre-Optic Area (POA). Behaviorally, I found that lizards in the Live Anole and Anole Video conditions did not differ in social display behaviors (pushups and dewlap extensions) or attentiveness, but lizards in both these conditions displayed more than lizards in the Scrambled Video and Control conditions – evidence that suggests there is no difference in lizard's behavioral responses to live lizards compared to video lizards. I also found evidence for the inhibitory nature of the POA, as the POA showed the least neural activity in the Live Anole condition, and there was a negative correlation between attentiveness and POA activity within the Live Anole condition. Finally, I saw no differences in LGN and NROT activity across the four treatments, providing evidence that lizards process

visual information in the visual brain regions independently of the social context of that information. Overall, this study provides a greater understanding of the behavioral similarities, but neural differences, in visual and social processing of a live anole compared to a video representation of an anole, suggesting caution in the use of video representations of behavior.

**Acknowledgements**

I would not have been able to complete this project without the immense amount of support provided to me by Trinity University faculty, students, and my friends and family. Above all, I would like to thank Dr. Michele Johnson for inspiring me to think outside the box and be accountable for my own project. Her attention to detail and level of knowledge have astounded me throughout my time working in her lab, and her desire to be a strong mentor for every single individual in the lab is commendable. Working so closely with her on this project, I feel I have learned more about science and the scientific process than in most of my Trinity classes. I would also like to thank Dr. James Roberts for being a supportive committee member, always willing and able to answer any questions I have and always willing to give constructive commentary on my hard work. Furthermore, I would like to thank Dr. Jonathan King and Dr. Gerard Beaudoin for assisting me in all my confocal needs. As I had never used a confocal microscope before, they were patient, helpful, and willing to answer my many questions. In addition to these many outstanding professors, I would like to thank Brittney Ivanov and Miguel Webber. From constructing the videos and running the trials, to developing an ICC protocol and executing it, both of these individuals have been integrally involved in my project and I could not have succeeded without them. I would like to thank Jake Stercula for helping catch lizards for the trials and assisting me in running the trials. I would also like to thank Faith Deckard, Adam Zeb, Emily Styslinger, and Leah Selznick for their help and support during the behavioral trials, Marzieh Rouzbehani and Jesus Vega for assisting with ICC, and Daisy Horr for assisting with analyzing behavioral data. Lastly, I would like to Logan Langford for his endless love and emotional support, even from 2,042 miles away, as well as my sister, Paula Andrea Jaramillo,

and my parents, Gloria Patricia Hoyos and Herman Horacio Jaramillo, for making my Trinity education possible.

This thesis was funded by Trinity University's Murchison Fellowship, Sigma Xi Grant-in-Aid-of-Research, the National Science Foundation (IOS 1257021 to M.A. Johnson), and Carey Wildlife Reserve.

## Introduction

Almost all animals rely on visual information to understand the world around them. For example, many animals use visual cues to facilitate social interactions such as territory defense, courting a potential mate, or determining the location of resources (e.g., birds: Rogers & Kaplan, 2000; amphibians: Hödl and Amézquita, 2001; insects: Lloyd, 1971). These complex cues are detected and processed in the brain, which then coordinates the animal's behavioral responses (Brattstrom, 1974). One type of response to social information is to perform a behavioral display, usually directed at one or more other individuals. Many aspects of a behavioral display, such as the color or motion components of the display, are highly stereotyped; however, within species individuals can differ dramatically in their display rates, combinations of various display components, and/or the context of the display.

To understand how animals respond to visual cues, experiments where stimuli are controlled are useful. However, because live animals are variable in how they behave in certain situations, it is difficult to use live animals as experimental stimuli. Video playback technology offers the potential to present visual animals with controlled stimuli, but can focal animals recognize a video as a social stimulus? In this thesis, I use expression of the immediate early gene c-*fos* as a measure of neuronal activity. I test whether video stimuli elicit similar responses in the brain as live stimuli in green anole lizards (*Anolis carolinensis*).

### *Anole Social Behavior*

The almost 400 known species of *Anolis* lizards are primarily distinguished by two traits: expanded toepads that allow the lizards to move on vertical surfaces, and a colorful throat fan

known as the dewlap (Losos & Schneider, 2009). *Anolis* lizards are diurnal (i.e., their periods of activity occur during the day), generally eat insects and other arthropods, and defend territories that overlap extensively with territories of the opposite sex. Anoles are tropical lizards, found mostly throughout the Caribbean, Central and South America, and the Southeast United States (Losos, 2009).

*Anolis carolinensis* (green anoles) communicate largely through visual cues (Jenssen, 1977), with little to none of their communication implementing chemical or auditory modalities (Robinson et al., 2015). Their visual displays, and the contexts in which these displays are used, have been described in detail (e.g., Greenberg, 1977; Jenssen et al., 1995). These aspects, along with the availability of a forebrain atlas (Greenberg, 1982), make green anoles an excellent model for studies of visual communication.

Visual displays used by green anoles include information communicated via motion, color, and postural changes (which during displays, often makes the lizard appear to be larger). Displays normally involve extension of the dewlap, head bobs, and pushups. Male anoles generally perform these displays more frequently than females, and most displays occur during courtship, territorial defense, or occasionally to deter predators (Leal & Rodriguez-Robles, 1997). Territorial displays may exhibit increasing intensity with the lizard raising themselves further from the ground as the interaction escalates (Jenssen et al., 1995). Escalation of territorial behavior also often results in the erection of nuchal or dorsal crests , and the development of a dark spot posterior to the eye (Greenberg, 1977; Jenssen et al., 2000). Male anoles in dispute will position themselves side-by-side with their heads oriented in opposing directions, sometimes circling around and lunging at each other as the competition progresses until one lizard retreats or attacks (Jenssen et al., 1995). This suite of visual displays allows anoles to assess competitors

prior to violent combat, or, in the context of courtship, to evaluate potential mates prior to copulation; thus, interpreting visual cues is of clear ecological importance for these animals.

### *Anole Visual Ecology*

The field of visual ecology examines animal visual systems in relation to their ecological needs. The sensory drive hypothesis, a major hypothesis in the field, predicts that animal sensory systems have evolved for effective animal functioning, and that different systems may function best in different environments (Fleishman, 1992). The visual system, the behavioral repertoire, and the environment must work together to form the anole sensory system.

Light conditions in an environment often affect the interpretation of a visual stimulus. For anoles, light conditions differ across habitat types with varying degrees of shade or sun; however, species with variation in light habitat conditions had little to no variation in spectral sensitivity, or in other words, the colors to which a lizard's eye is most sensitive (Fleishman et al., 1997). Spectral sensitivity is dependent on the types and quantities of photoreceptors present in the retina, and while different species of anoles have different numbers and types of photoreceptors, their spectral sensitivities do not vary; all anole species exhibit consistent spectral sensitivity function with peak sensitivity at 550 nm – the spectral radiance of vegetative backgrounds (Fleishman et al., 1997). This means that spectral sensitivity is more dependent on the habitat background color (which does not vary substantially across species) than the habitat light conditions (which do vary among species).

Each green anole eye has a 180° monocular receptive field, and the lateral placement of the eyes on each side of the head allows for 20° of forward-facing binocular overlap: a broad

field of view critical for the anole's ability to scan for prey as it sits motionless on a perch (Fleishman, 1992). Designed for high-acuity diurnal vision, the retina of the anole eye is unique in its photoreceptor composition and density across the peripheral retina, temporal fovea, and central fovea. Photoreceptors of the retina are all of the cone type, with no rods present, and the density of cones in the central fovea is ten times the density of cones in the peripheral retina (Makaretz & Levine, 1980), while the temporal fovea, associated with binocular fixation, has photoreceptor densities that are lower than the central fovea but still three times greater than in the peripheral retina (Fite & Lister, 1981; Fleishman, 1992). The peripheral retina has a retinal ganglion cell (RGC) to cone ratio of 1:1; however, in the central fovea this ratio declines. The absence of rods and this low RGC to cone ratio lead anoles to have high acuity vision in high-intensity light environments while having low acuity vision in low-intensity light environments.

Green anoles have a total of four cone types whose absorption maxima are approximately 565, 495, 450, and 365 nm (Leow & Fleishman, 1993). The cones with absorption maxima at 565, 495, and 450 nm are visible light spectrum-sensitive, while the cone with absorption maxima at 365 nm is an ultraviolet-sensitive cone. The ability for ultraviolet vision is particularly important in anole visual communication through the use of ultraviolet-reflective dewlaps in ultraviolet-rich habitats (Leow & Fleishman, 1993). Stoehr and McGraw (2001) investigated coloration and ultraviolet reflectance of dewlaps in *Anolis carolinensis* and found that green anole dewlaps reflect maximally in both UV and long-wavelength portions of the light spectrum. In addition, green anole dewlaps are highly exposed to UV light in open spaces (Stoehr & McGraw, 2001), suggesting a selective advantage for green anoles to reflect UV light signals from their dewlap in UV-rich environments. Stoehr and McGraw (2001) also postulated that the ability for the green anole dewlap to reflect light of both UV and long-wavelength light

allows for visual communication that is accurate and efficient in environments that are sunny or cloudy. Combining information from both dewlap reflectance with visual perception, green anoles not only reflect light of both spectrums, but they perceive them as well. This link between behavior and the animal's visual system sheds light on the evolution of visual signaling among green anoles.

Visual ecology makes it increasingly apparent that visual cues rely on habitat background and light reflectance. This leads to concern for the use of video technology in behavioral experiments as videos often fail to incorporate background cues from an ecological context – or to fully represent the natural interaction of the dewlap with light – a potentially critical component of dewlap function.

## ***Visual and Social Processing in the Brain***

### *Visual Pathways*

Following the detection of visual and social cues by the eye, these cues are then processed and interpreted in the brain. There are three pathways (Figure 1) that project visual information from the anole retina to the telencephalon (the largest and foremost division of the brain): the lemnothalamic pathway, the collothalamic pathway, and the retino-thalamic pathway (Bruce, 2009). The lemnothalamic pathway projects from the retina to the lateral geniculate nucleus (LGN) of the thalamus and then to the primary visual cortex (Hodos and Butler, 1997). The collothalamic pathway projects from the retina through the optic tectum to the nucleus rotundus (NROT) of the thalamus, and then to the visual nuclei of the dorsal ventricular ridge (DVR) and the striatum (Hodos and Butler, 1997). The third pathway, the retino-thalamic

pathway, projects from the retina, through the optic tectum to the LGN of the thalamus and then to the DVR and/or pallial thickening (Bruce and Butler, 1984). While the retina is always the first to receive the visual input, the optic tectum serves as an intermediary between the thalamus (LGN and NROT) and the visual cortex (DVR/Pallial thickening/striatum) in the retino-thalamic and collothalamic pathways.

The mammalian visual system is in many ways homologous to the reptilian visual system. Information processed in the mammalian visual system travels through the retina to the optic chiasm and then up to the LGN of the thalamus where it is directed to the primary visual cortex (V1) (Breedlove et al., 2013). This mirrors the reptilian visual system nearly identically. For example, both reptilian and mammalian systems have pathways travelling from the retina to the LGN; however, V1 and DVR/pallial thickening/striatum, while homologous, present unique variations in the processing of visual information. In mammals, perception of information is not restricted to V1, but actually occurs throughout the visual processing pathway. It is unclear the extent to which visual processing occurs throughout this pathway in reptiles.

*Social Behavior Network (SBN)*

Social information is processed in a different suite of brain regions. Newman (1999) and Goodson (2005) described the social behavior network (SBN) as six brain nuclei that occur in all vertebrates, and interact with each other to process social information and therefore elicit a behavioral response. These six brain nuclei include the amygdala, the lateral septum, ventromedial hypothalamus, anterior hypothalamus, preoptic area (POA), and the midbrain (Figure 2). Goodson and Kabelik (2009) described the relationship between vertebrate social behaviors and activation of the SBN. Behavior is most strongly associated with patterns of neural

activity across nuclei of the SBN rather than with one single nucleus, resulting in endless combinations of neural activity that can be associated with particular nuclei (Goodson & Kabelik, 2009). Likewise, work on social cues and the SBN has shown that social stimuli differentially activate hypothalamic networks, and that social experiences affect social behavior through the modification of SBN brain regions (Hoke et al., 2005; Yang & Wilczynski, 2007).

These brain regions have also been shown to be implicated in social behavior in the green anole. Greenberg et al. (1984) showed that lesioning of the amygdala and paleostriatum in the green anole resulted in reduced aggressive and courtship displays while lesioning only the amygdala impaired courtship behaviors and lesioning only the paleostriatum impaired challenge behaviors. Further, Tarr (1977) lesioned the medial amygdala in the western fence lizard, *Sceloporus occidentalis*. Normally, *S. occidentalis* demonstrates a predictable and consistent social display across environmental and social situations, but lesioned lizards failed to assert dominance or subordinance behaviors, suggesting the amygdala's critical role in socially mediated aggression. In addition, findings by Neal and Wade (2007) suggest that the POA is inhibitory in the context of social behavior, while findings by Morganteler and Crews (1978a, b) suggest that the POA is implicated in regulating reproductive behavior in green anole lizards. By lesioning the anterior hypothalamic-preoptic area, Morgantaler and Crews (1978a) abolished courtship and agonistic behaviors in male green anole lizards that were not castrated and in those that were castrated and treated with androgens. In a similar study, Morgantaler and Crews (1978b) used testosterone implants in the anterior hypothalamus-preoptic area to restore reproductive behavior in castrated anoles. Beck et al. (2008) found differences in limbic brain region structure between male and female green anoles, and between breeding and post-breeding seasons. Their findings suggest that the limbic brain regions (POA, amygdala, and ventromedial

hypothalamus) are dynamic and that there are parallels between morphology of the POA and anole expression of masculine behaviors.

### *Connecting the Visual Pathways and the SBN*

The SBN and visual pathways must be connected for animals to perceive visual signals as socially relevant and therefore elicit a behavior in response. In the Algerian sand lizard (*Psammodromus algirus*), this connection has been found to project from the LGN to the NROT and then to the DVR and the amygdala (Guirado et al., 2000). This axonal connection likely bridges the social behavior network to the visual pathways.

## ***Overview of the Current Study***

### *Behavioral Trials*

In this study, I examined green anoles to determine neuronal activation within nuclei implicated in visual processing and nuclei implicated in social behavior when observing live anoles compared to video anoles. I manipulated visual stimuli that elicit highly social behavioral responses and measured the resulting changes in activity within the nuclei of the brain. I analyzed the nuclei of the POA for social behavior (Newman, 1999; Goodson, 2005) and the NROT and LGN for visual processing (Manger et al., 2002; Bruce, 2009).

I used a series of four behavioral treatments, in which each lizard experienced a single trial, to manipulate social and visual experience The four trials included a Live Anole condition where the visual and social stimuli were from another live anole, an Anole Video condition where the visual and social stimuli were a recorded video of an aggressive lizard display, a

Scrambled Video condition where the visual stimulus was the aggressive dewlap display video with the pixels scrambled to remove the lizard image, and a Control condition where the visual stimulus was a perch, with no obvious social stimulus. According to Macedonia et al. (1994), video-recorded sequences of lizard displays are sufficient to mimic behavioral responses expected in a live interaction. Studies since have used video playback as a plausible method of controlling for confounding variables while eliciting the desired behavioral response in lizard animal communication (e.g., Ord et al., 2000). By looking at brain activity during video exposure, we can determine the accuracy of video representations of anole behavior, as perceived by the anole viewing the videos.

*Quantifying Neuronal Activity*

In humans, fMRIs use blood oxygen levels to quantify brain activity in certain nuclei; however, this technique is quite expensive and is not available for all animals. On the other hand, an immediate early gene, c-*fos*, is a gene transcribed within minutes of neuronal activation, and quantification of c-*fos* is a useful tool for identifying brain activity. In this study, I measured levels of c-*fos* protein as a marker for neuronal activity in the brains of the lizards in each of the four experimental conditions (Morgan & Curran, 1991; Cruz et al., 2013).

Activation of the immediate early gene c-*fos* occurs within a few minutes of growth factor stimulation and is not detectable after 30 minutes (Sheng & Greenberg, 1990). This time delay is an advantage as it allows the animal to be handled immediately before euthanasia without interfering with the c-*fos* signal. The c-*fos* gene encodes for the c-*fos* protein which has a leucine zipper motif allowing for dimerization with members of the Jun family of oncogenes (c-Jun, Jun B, and Jun D) on the AP-1 binding domain of DNA (Hoffman et al., 1993). Due to

DNA binding, staining for c-*fos* is localized to the cell nucleus; therefore, detection of c-*fos* can be accomplished with standard double labelling techniques such as immunocytochemistry (Hoffman et al., 1993). This DNA binding also allows c-*fos* to function in a way that rapidly alters gene transcription, and c-*fos* gene expression is induced in neurons following neuronal stimulation (Hoffman et al., 1993).

I used immunocytochemistry to quantify levels of c-*fos* which allows the measurement of activation within specific areas of the brain following the behavioral trials (following Guzowski et al., 2005; Neal and Wade, 2007). Using patterns of c-*fos* expression in the brain, I explored the connection between visual signals during visual exposure to live and video anoles and the activity of the brain regions that process those signals.

To understand visual processing of the aggressive video sequence in the green anole brain, I measured c-*fos* in the NROT and LGN. By analyzing the activity of these visual regions I hope to not only identify activity in these regions but also distinguish the pathways through which certain visual stimuli are processed. If activity is enhanced in the NROT, visual signals are being preferentially processed in the collothalamic pathway. If activity is enhanced in the LGN, visual signals are being preferentially processed in the retino-thalamo-telencephalic pathway or lemnothalamic pathway. In the SBN, I measured c-*fos* in the POA due to its well-defined roles in processing social behavior in anoles (Newman, 1999; Goodson, 2005; Manger et al., 2002; Bruce, 2009).

***Hypotheses***

First, I tested the hypothesis that stimuli with the most behavioral information will elicit the strongest behavioral response from lizards. I predicted the greatest behavioral response from anoles in the Live Anole condition, the second most behavioral response in the Anole Video condition, and the least behavioral response in both the Scrambled Video and Control conditions. Next, I tested the hypothesis that the visual and social nuclei of the lizard brain will have a high integrated density of c-*fos* when exposed to another anole, and that these nuclei will have a low integrated density of c-*fos* when given no visual or social stimulus.

Last, I tested the hypothesis that neuronal activation of visual and social nuclei are associated with lizards' behavioral responses to social cues. I tested for correlations between attentiveness, dewlap display, and pushup behavior with the mean integrated density in each brain region to see if behavior correlates with cerebral response. I predicted that high levels of behavior will correlate with high levels of integrated density in visual brain regions, in each condition, while because of the likely inhibitory function of the POA (Neal & Wade, 2007), high social behavior will be associated with low activation of the POA.

## Methods

### *Study Organisms and Animal Care*

Forty adult male *A. carolinensis* were captured by hand or noose from natural areas in San Antonio, Texas in July 2015. Anoles were individually housed in Trinity University's Animal Care Facilities in clear, plastic cages (27 x 21 x 14 $cm^3$) for no more than 4 days prior to experimental manipulation. Each cage contained R'zilla terrarium liner (Zilla, Franklin, WI) and a natural wooden perch, and light was provided by 25W full spectrum UV heat lamps (Zoo Med Laboratories, Sacramento, CA) set to a 12:12 L:D cycle. A wooden board was placed between cages to prevent visual contact between males. Temperatures in the facility ranged 25.3-28.4°C and humidity ranged 56-75%. Anoles were misted daily and fed a diet of 1-2 crickets coated in Fluker's calcium powder (Port Allen, LA) every other day.

Lizards were randomly assigned to each treatment condition. To ensure that each condition did not differ in body size or head dimensions (traits that are associated with dominance behaviors (Bush et al, 2016), for each lizard, I measured snout-vent length (SVL) using a clear plastic ruler, mass with a Pesola spring scale, and head dimensions with digital calipers. I measured head width as the distance from ear to ear (the widest part of the skull), head length rostro-caudally from the tip of the mouth to the parietal eye, and head depth dorsal-ventrally from the parietal eye to the bottom of the jaw (the deepest part of the skull). Trinity University's Animal Research Committee approved all procedures used in this study (protocol # MJ050616 and 011415_MJ1).

### *General Experimental Methods*

I randomly assigned ten lizards to each of the four conditions of behavioral trials and trials: Live Anole, Anole Video, Scrambled Video, or Control. All trials were run in random order. Lizards in the four conditions did not differ significantly in mass, SVL, or relative head length (calculated as the residuals from a regression of head length vs. SVL; Table 1: ANOVAs all $F < 1.12$ and $p > 0.35$). Each trial consisted of putting a lizard in a visually neutral arena, presenting it with visual information from another live anole or from carefully constructed video playback (Macedonia and Stamps, 1994; Yang et al., 2001), and recording its behavioral responses.

### *Video Production*

To produce the videos used in behavioral trials, I captured two adult male anoles from the grounds of Trinity University, placed them both in a mesh cage (20 x 25 x 15.5 $cm^3$) that contained two GoPro HERO 3 white edition cameras (San Mateo, CA), and allowed the lizards to interact. When the lizards performed aggressive behaviors, I recorded these behaviors and then used this footage in the trial videos. I produced three separate streams of footage: a 15 min sequence of aggressive behavior (aggressive footage), that 15 min footage of aggressive behavior with the pixels scrambled (scrambled footage), and a 10 min and 25 min still frame sequence of a perch with no lizard (lizardless perch frame footage).

*Aggressive Footage*

To produce the aggressive footage, I imported approximately 60 sec (40 sec of a male anole's aggressive push-up and dewlap display and 20 sec of the same anole performing only minor head turns) into Adobe Premiere Pro CC. Color, brightness, and sharpness were adjusted to best mimic the appearance of a real anole. The footage was then cropped to fit the size of an iPad screen and to approximate the actual size of a large *Anolis carolinensis* male. Next, the background was erased using repeated applications of a 16 point garbage matte that allowed us to mask out everything but the lizard and its perch, leaving behind a white background that matched the behavioral trial arena's white walls. I exported this approximately 60 sec display-rest-display sequence as a series of still frames, at a standardized rate of 30 frames per second, which I then imported into Adobe After Effects CC, where I set each frame to run for 1/30 sec and exported it as a video of the same length as the original imported footage. I then imported this aggressive sequence into Premiere Pro, where these sequences were then alternated and looped with smooth transitions to produce 15 min of an aggressive, intermittent lizard display composing the aggressive footage.

*Scrambled Footage*

To produce the 15 min of scrambled footage, I collaborated with Charles Stein ('17) of Trinity University's Department of Computer Science who wrote a pixel randomizer code in Matlab designed to scramble the pixels in the portion of the aggressive video that included the anole. The code works by partitioning each image of the video to isolate a rectangle containing the entire lizard. This rectangle has an x, y and z axis. The x and y axes correspond to each pixel

of the image while the z corresponds to the color – red (R), green (G), or blue (B). I permuted only the x and y axes, allowing us to write the code so the color integrity of the pixels was preserved.

I then followed all the steps used in producing the aggressive footage with a single additional step: before importing the footage into Adobe After Effects CC, I ran all the frames through the pixel randomizer. By using this code, I was able to maintain the same pixel colors and image complexity while ensuring that the dewlapping lizard was no longer clear in the video.

*Lizardless Perch Frame Footage*

To produce the lizardless perch frame, a still frame of the lizard was captured from the aggressive footage and exported into Adobe Photoshop CC, where the anole was digitally removed but the image of the perch remained intact. I then imported this frame sequence into Premiere Pro, where it was looped with smooth transitions to produce 10 min of a lizardless perch comprising the acclimation period, and to produce 25 min of a lizardless perch comprising the nonsocial control video.

Using the unscrambled aggressive footage, scrambled footage, and lizardless perch frame footage, three separate videos were created in Premiere Pro and exported to YouTube, where they were streamed to an iPad (Apple, Cupertino, CA) for use in the behavioral trials.

***Behavioral Trials***

The arena consisted of a 61 by 30 by 30 $cm^3$ plastic container with a lid and a wooden perch (Figure 3). The container and lid were both painted with white spray paint (Liquitex, Cincinnati, OH). The perch consisted of a circular wooden rod with a diameter of 2 cm and a length of 61 cm. I drilled 3 circular holes, each 2.5 cm in diameter, and one rectangular hole at a height of 13.5 cm and a width of 19 cm, into different faces of the arena. I used two of the circular holes for the two GoPro HERO 3 cameras, one circular hole for the perch, and the rectangular hole for the iPad screen. One of the circular GoPro camera holes was drilled above the rectangular hole on one of the 30 cm faces of the container while the other circular GoPro camera holes was drilled halfway up one of the 61 cm faces of the container. The final circular hole was used to insert the perch, at an approximately 30° angle, into the arena. I drilled the final circular hole on the final 30 cm face, opposite the face with both the iPad and the GoPro camera holes. I tethered the anole to the perch, using a dental floss, slip-knot noose tied around the lizard's abdomen, in each trial and placed it into the arena oriented towards the iPad screen. In the arena, I exposed the lizard to a 10 min acclimation period, followed by 15 min of exposure footage played on an iPad (either aggressive footage, scrambled footage, or lizardless perch frame), or 15 min of exposure to another live anole. I recorded the lizards' behaviors (number of head turns, licks, movements, times the lizard fell off the perch, dewlap extensions, and pushups; and duration of dewlap extensions and time not facing iPad screen) during each trial using the GoPro cameras. Within 5 sec of completion of each trial, the lizard was euthanized via rapid decapitation. I then immediately dissected out the lizard's brain, which was flash-frozen in isopentane within at most 5 min after trial completion. All tissues were stored at -80°C until further processing.

*Live Anole*

The Live Anole condition consisted of two anoles in the arena, processing visual information from each other. In this condition, both lizards were focal animals, and both were included in all subsequent measures. I tethered all lizards, as described above, to ensure visual and social perceptions were not affected by any possible physical contact between the anoles and to ensure consistency amongst all conditions. For the 10 min acclimation period, I prevented visual contact between the lizards using a 8.5 x 11 $in^2$ printed paper copy of the lizardless perch frame inserted into a clear self-standing desk frame. This ensured that lizards in all conditions had similar visual stimuli during the acclimation period. Following acclimation, I removed the frame, and the lizards were allowed to interact for 15 min.

*Anole Video*

The Anole Video condition consisted of an anole in the arena, tethered to a perch, exposed to standardized video playback of an aggressive anole display. This video included 10 min of lizardless perch frame footage (i.e., the acclimation period) followed by 15 min of the aggressive footage.

*Scrambled Video*

The Scrambled Video condition consisted of an anole in the arena, processing visual information from standardized video playback of an aggressive lizard display where the pixels had been scrambled. This video included the 10 min of lizardless perch frame footage (i.e., the acclimation period) followed by 15 min of the scrambled footage.

*Control*

The Control condition consisted of an anole in the arena with standardized video playback consisting of 25 min of the acclimation period lizardless perch frame footage.

***Immunocytochemistry***

I coronally cryosectioned each brain in four alternate series at 20 µm and thaw-mounted each section onto SuperFrost Plus microscope slides (Fisher Scientific; Hampton, NH). I stored all slides at -80°C until further processing.

I used immunocytochemistry to measure brain activity using the immediate early gene c-*fos* (Guzowski et al., 2005; Neal & Wade, 2007). Alternate slide series (i.e., those sections at 40 µm intervals) were warmed to room temperature. I fixed tissue in 4% paraformaldehyde for 10 min followed by three 5 min rinses in PBS. I then incubated the slides for 2 h in 4% normal donkey serum (EMD Millipore, Temecula, CA), 0.1M PBS, and 0.3% Triton X-100. Following this initial incubation, I incubated tissues in c-*fos* primary antibody (1:1000 EMD Millipore, Temecula, CA) in 0.1M PBS with 0.3% Triton X-100 at 4°C for 48 h. After 48 h, I rinsed tissues in PBS 3 times for 5 min each time. In the dark, I incubated the tissues in donkey anti-sheep secondary antibody (1:1000 ThermoFisher Scientific, Waltham, MA) for 2 h. I then rinsed the tissues in 0.1M PBS 3 times for 5 min each time before coverslipping with DAPI fluoromount-G (Southern BioTech, Birmingham, AL). I stored tissues for a minimum of 1 d in a light proof box before quantifying c-*fos* levels in the POA, NR, and LGN using a confocal microscope.

In order to confirm the absence of non-specific labeling, I used two controls. First, I processed tissues without primary antibody, and the detection of fluorescence within neurons

was low (see Results). Second, I ran a preadsoprtion control by mixing the c-*fos* primary antibody (EMD Millipore, Darmstadt, Germany) with 20 times molar excess c-*fos* synthetic peptide (ThermoFisher Scientific, Waltham, MA) and observed minimal fluorescent signal (see Results).

### *Confocal Imaging*

I imaged slides at 40X magnification on a Nikon A1 Confocal microscope (Nikon Instruments). I used a DAPI and TRITC laser with parameters optimized to detect AlexaFluor 555: 402.6nm (DAPI) laser power 1.0, gain 120, offset -3; 561.4nm (TRITC) laser power 3.0, gain 130, offset -7. I standardized my capture settings by adjusting the settings (laser power, gain, and offset) on a single run so that I was using the full width of the histogram for both DAPI and AlexaFluor 555. I chose slides with the highest c-*fos* expression so that the signal on all slides would fall within the confocal's range limitations. The pinhole was always set to 3.2. For capture, all lasers were fired in a channel series. All images had an optical resolution of 0.2µm and an optical sectioning of 0.33 µm. I used 4x line averaging to reduce background signal.

I captured 2-6 images located in the rostrocaudal center of each brain region for each hemisphere (left and right). In the NROT, I measured nuclei in one 204.8 µm x 204.8 µm. This yields a total of 41,943 $\mu m^2$ per image. In the POA, I measured nuclei in one 184.3 µm x 92.2 µm. This yields a total of 16,986 $\mu m^2$ per image. In the LGN, I measured nuclei in one 204.8 µm x 51.2 µm. This yields a total of 10,486 $\mu m^2$ per image.

***Data Analysis***

I quantified c-*fos* levels using ImageJ. I used a macro that identified all the nuclei in the image, then analyzed fluorescence levels in each nucleus. The program measured the area of the nucleus, mean levels of fluorescence in each nucleus, integrated density (the area of the nuclei multiplied by the mean fluorescence within the nuclei), and raw integrated density (the sum of the values of the pixels within the nuclei). In subsequent analyses, I used integrated density as an estimate of c-*fos* expression. As a value that measures all the fluorescence within the nucleus and then multiplies by the area of the nucleus, the integrated density quantifies fluorescence in a way that controls for the area of nuclei in question. This is opposed to the mean fluorescence which does not take into account the size of the nuclei. I averaged all the results (from all pictures of both left and right sides) for a single brain region for each anole.

I performed a series of one-way ANOVAs to compare behavioral responses (attentiveness, dewlaps, pushups, times oriented body away from the stimulus, head turns, falls, licks, and movements) across the conditions. If no lizards in the condition performed the behavior, that condition was excluded from the ANOVA. Significant results were followed by Tukey's post hoc tests. Furthermore, I performed another series of one-way ANOVAs to compare neural activity (mean integrated density) for each region (POA, NROT, or LGN) across all four conditions. Significant results were followed by Tukey's post hoc tests. While behavioral analyses had sample sizes of 8-10 per condition, confocal image analyses (to date) had sample sizes of 3-5 per condition. To see if behavior was related to activity in associated brain regions I used a series of bivariate correlation analyses comparing attentiveness and each brain region: one with the entire sample and one with the sample split by condition. All analyses were performed in SPSS.

## Results

### *Behavior*

Lizards in the Live Anole, Anole Video, and Scrambled Video conditions were more attentive to the video (looked more at the screen, and did not turn away from the screen as often) than those in the Control condition (ANOVA: $F(3,35)=5.461$, $p=.004$, Figure 4). Conditions also differed significantly in number of times they oriented their body away from the stimulus (ANOVA: $F(3,35)=3.115$, $p=.039$) with lizards in the Live Anole condition turning away significantly less than those in the Control condition (p=.038) and lizards in the Anole Video condition turning away marginally less than those in the Control condition (p=.086). Conditions did not differ significantly in number of head turns (ANOVA: $F(3,35)=0.871$, $p=.493$), falls off the perch (ANOVA: $F(3,35)=0.853$, $p=.254$), licks (ANOVA: $F(3,35)=0.853$, $p=.475$), or movements (ANOVA: $F(3,35)=0.696$, $p=.561$) where a movement constituted a lizard moving three or more limbs (such as moving forward or backwards on the perches).

Social behaviors also differed among the four conditions, as lizards in the Live Anole and Anole Video condition performed both pushups and dewlap extensions, but lizards in the Scrambled Video condition performed only dewlap extensions, and lizards in the Control condition performed none of these displays. Live Anole and Anole Video conditions did not differ significantly in number of pushups performed (ANOVA: $F(1,18)=2.073$, $p=.153$, Figure 5) or dewlaps performed (ANOVA: $F(2,27)=2.306$, $p=.119$, Figure 6) .

### *Neural Activity*

The no-primary and preabsorption controls both showed minimal levels of integrated density, indicating a lack of non-specific binding of the antibodies (Figure 7). Lizards did not differ significantly across condition in area of neuron cell bodies in any of the three focal brain regions (ANOVA for POA: $F(3,12)=0.486$, $p=.699$; ANOVA for NROT: $F(3,10)=0.289$, $p=.833$; ANOVA for LGN: $F(3,11)=1.222$, $p=.348$).

Within the POA, the four conditions differed significantly in integrated density (ANOVA: $F(3,12)=4.602$, $p=.023$, Figure 8) with lizards in the Live Anole condition expressing less c-*fos* than lizards in the Scrambled Video condition (p=.019), and lizards in the Control condition expressing marginally less c-*fos* expression than lizards in the Scrambled Video condition (p=.065). Within the NROT and the LGN, there was no significant difference in integrated density among the four conditions (ANOVA for NROT: $F(3,10)=.545$, $p=.662$, Figure 9; ANOVA for LGN: $F(3,11)=1.245$, $p=.340$, Figure 10).

### *Bivariate Correlations*

I found a significantly positive correlation between integrated density in the two visual regions, the NROT and the LGN (r=.939, p<.001, Figure 11). Expression of c-fos in the social region, the POA, was not correlated with expression in either visual region.

With each condition considered separately, within the Live Anole condition, I found a marginally significant negative correlation between attentiveness and integrated density in the POA (r=-.935, p=.065, Figure 12). No other significant correlations were found between c-*fos* expression in the brain region and behavior.

**Discussion**

In this study, I found that lizards presented with a visual stimulus (i.e., those in the Live Anole, Anole Video, and Scrambled Video conditions) were more attentive to their visual cues than those with no visual stimulus (such as the Control condition). Furthermore, lizards in the Live Anole and Anole Video conditions did not differ in social display behaviors (pushups and dewlap extensions), but lizards in both these conditions displayed more than lizards in the Scrambled Video and Control conditions. I also found that in the social brain region (the POA) lizards exposed to another live lizard expressed the lowest amount of c-*fos*. Further, among lizards in the Live Anole condition, I found a strong negative correlation between attentiveness and c-*fos* expression. Together, these results suggest that social experiences result in decreased level of neural activation in the POA, consistent with an inhibitory role for the POA in facilitating social behavior. On the other hand, in the NROT and LGN there were no statistically significant differences in c-*fos* expression levels between conditions. Interestingly, there was a strong positive correlation between the NROT and the LGN (both regions of the visual pathway). The implications of these results are explored below.

I predicted that stimuli with the most behavioral information (such as the Live Lizard and Anole Video conditions) would elicit the greatest level of behavioral response and that behavioral response would decrease from Live Anole, to Anole Video, to Scrambled, and Control conditions. Dewlap behaviors (Figure 6) followed this predicted trend, while pushup behaviors followed the trend but less closely (Figure 5). In particular, lizards in the Scrambled Video condition performed dewlap behaviors, but never paired with pushup behaviors. This suggests that these dewlap behaviors observed in the Scrambled condition were spontaneous rather than social as displays in response to social stimuli generally include pushups, or dewlaps

and pushups in combination, but not dewlaps alone; therefore, they were likely not in response to any particular behavioral stimulus coming from the video (Greenberg, 1977; Jenssen, 1977). With no significant differences in behavior between the Live Anole and Anole Video conditions, these data support the generality of Macedonia et al.'s (1994) and Ord et al.'s (2000) findings that video stimuli offer sufficient behavioral information to mimic the behavioral response to a live animal. Similar findings have also been observed when exposing anoles to a robotic representation of a male anole (Martins et al., 2005). Together, similarity in behavior between video and live representations as well as robotic and live representations supports the use of alternative representations of anoles to successfully mimic live anoles, at least behaviorally.

Attentiveness also followed my predicted trend; however, the Scrambled Video condition showed a greater level of attentiveness than expected. Lizards in the Scrambled Video condition may have displayed a high level of attentiveness to the movement on the screen if that movement was similar to movements that lizards would attend to in an ecological context, such as prey. As sit and wait predators, anoles hunt by waiting and scanning for prey in a particular location (Moermond, 1979). If the lizards in the Scrambled Video condition were scanning for prey, this would have resulted in attentiveness to any movement. Another possible explanation for this level of attentiveness is that the movement on the screen was similar to movements in a second ecological context – predators. Anoles often implement a survey posture when scanning for predators (Stamps, 1977). Similar to scanning for prey, this survey behavior in response to unexpected and unfamiliar movement likely results in a greater level of attentiveness to the movement thereby preparing the lizard in case of a threat. In this experimental condition, the only representation of movement in the arena came from the video; therefore, any lizards scanning for prey or predators in the Scrambled Video condition would have been attentive to

the video. This offers a plausible explanation for the similarity in attentiveness between the Scrambled Video condition and the two conditions in which high attentiveness was expected because of the presence of another lizard (live or video).

Furthermore, I predicted that there would be a direct association between levels of activity in the brain nuclei, behavioral responses to social cues, and exposure to another anole. As predicted, I found that the Live Anole condition had the lowest levels of c-*fos* expression in the POA while the Scrambled condition had the highest levels of c-*fos* expression in the POA. The lower levels of c-*fos* expression in the Live Anole condition are consistent with findings by Neal and Wade (2007) that greater levels of social exposure correlate with lower levels of c-*fos* expression in the POA. Further, the negative correlation between POA activity and attentiveness in the Live Anole condition indicated that the POA had greater c-*fos* expression when the lizard was less attentive. Together, these results further suggest that the POA may act as an inhibitory structure, and shutting it off in the social pathways ensures greater expression in other regions of the SBN that will further process highly social responses. The inhibitory nature of the POA has also been previously observed in mammals (McIntyre et al., 2002; Simmons and Yahr, 2003). In relation to video, finding a correlation between attentiveness and POA activity in the Live Anole condition and not the Anole Video condition brings into question whether the Anole Video is truly mimicking the Live Anole during processing of visual and social stimuli, although not all brain tissue samples have yet been analyzed.

I predicted that both visual regions of the brain would exhibit enhanced activity following social stimuli exposure; however, there were no significant differences in c-*fos* expression in the NROT or LGN across conditions. The similarity in activity in the NROT and LGN between the different conditions suggests that while the lizards are processing the images in the visual

pathways, all images are processed in a similar way with no regard to which images are more socially relevant than others. This correlation also suggest that multiple visual pathways (Figure 1) are used simultaneously. Studying primates, Joffe and Dunbar (1997) described visual brain regions as devices for input of social information from socio-visual stimuli (which includes facial expressions and bodily gestures). On the other hand, Joffe and Dunbar (1997) claimed the social brain regions of the neocortex serve to encode and interpret these social cues following the initial input by the visual brain regions. They found a correlation between social group size of the animal and the V1 visual area; however, this correlation is not present in non-V1 visual brain regions (such as the LGN). Interestingly, in primates the LGN projects to the V1 visual area (Mignard & Malepli, 1991). In anoles, the LGN projects to the dorsal cortex/pallial thickening. If neural activity in anoles mimics neural activity in primates, these forebrain regions of the anole may exhibit a greater level of social processing than the LGN and NROT (Figure 1). Similarly, in humans recognition of familiar faces has been found to occur in the visual and social brain regions with activity in the extrastriate visual cortex exhibiting greater activity when processing the most familiar faces (Gobbini & Haxby, 2007). My findings in green anoles show that processing of the social information within the image does not occur during the early visual processing of the image, but may occur either later in visual or processing or after visual processing is complete. Additionally, activity in neither the NROT nor LGN correlated with activity in the POA. While there is a connection between the visual pathways and the SBN (Guirado et al., 2000), this result implies that there are still differences in the inputs that these regions receive and/or how these regions process their inputs. This leads to the conclusion that the visual and social pathways, while connected, remain fairly independent in processing.

To address the question “do videos accurately mimic live interactions?” this study provides results from brain and behavior. Firstly, evidence from this study supports the findings from previous studies (Macedonia et al., 1994; Ord et al., 2000) that lizards do not behave differently when exposed to a live lizard compared to a video representation of a lizard. In addition, socially relevant images do not result in greater activity in the visual pathways than non-socially relevant images. In terms of neural processing of videos in comparison to live interactions, attentiveness and POA activity had a strong negative effect in the Live Anole condition only, suggesting differences in neural processing between the Anole Video and Live Anole conditions. While these results do not discount the use of video in studying animal behavior in a controlled environment, they do suggest caution in the use of video stimuli in lizard behavioral experiments. Further analyses involving all the relevant visual and social brain regions (in addition to the NROT, LGN, and POA) will allow us to better understand whether or not videos mimic neural processing of live interactions, and if not which brain regions differ in processing.

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**Table 1.** Mean and standard deviation of mass, SVL, and residuals of Head Length: Snout-Vent Length (HL:SVL) for each condition

| | **Mass** | | **SVL** | | **Residuals of HL:SVL** | |
|---|---|---|---|---|---|---|
| **Condition** | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* |
| *Live Anole* | 3.82 | 1.48 | 64 | 3.62 | -.06 | .92 |
| *Anole Video* | 4.99 | 1.96 | 65.6 | 5.97 | -.30 | .99 |
| *Scrambled Video* | 4.98 | 1.91 | 65.3 | 5.31 | .27 | 1.30 |
| *Control* | 4.62 | 1.94 | 64.8 | 7.33 | .06 | .87 |

**Figure 1.** Visual pathways projecting visual information up to the telencephalon. Nuclei circled in orange are the visual nuclei of interest in this study. Figure adapted from Bruce (2009).

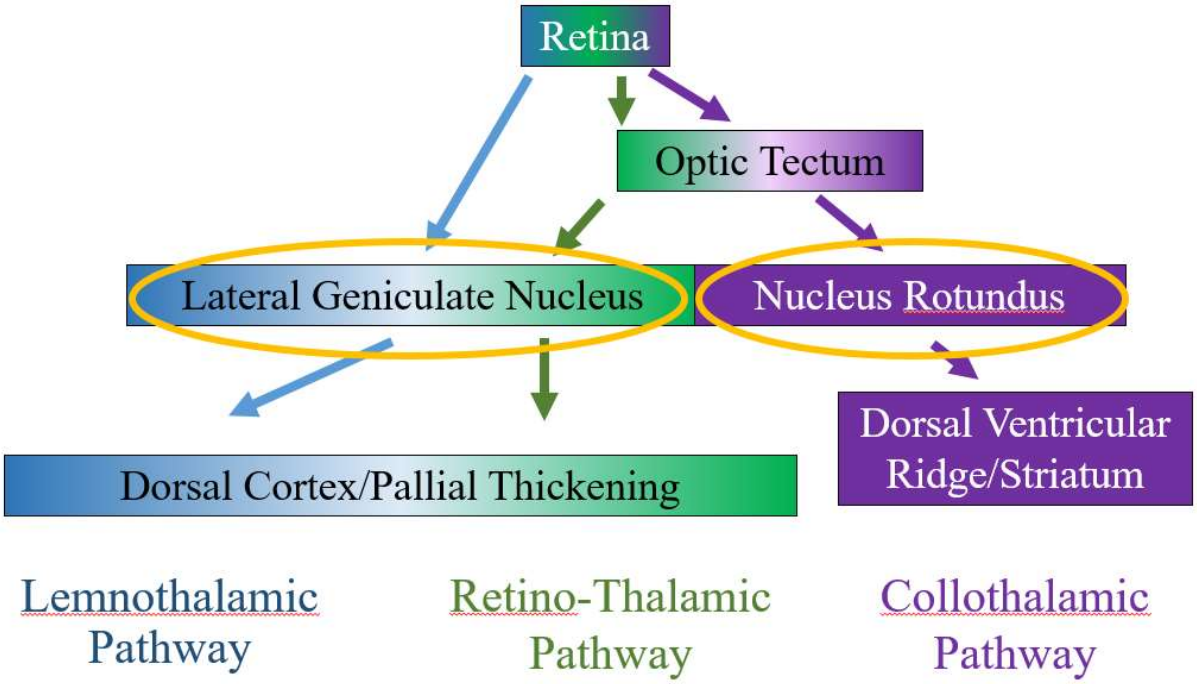

**Figure 2.** The social behavior network. Nuclei circled in orange are the social nuclei of interest in this study. Figure adapted from Goodson, 2005.

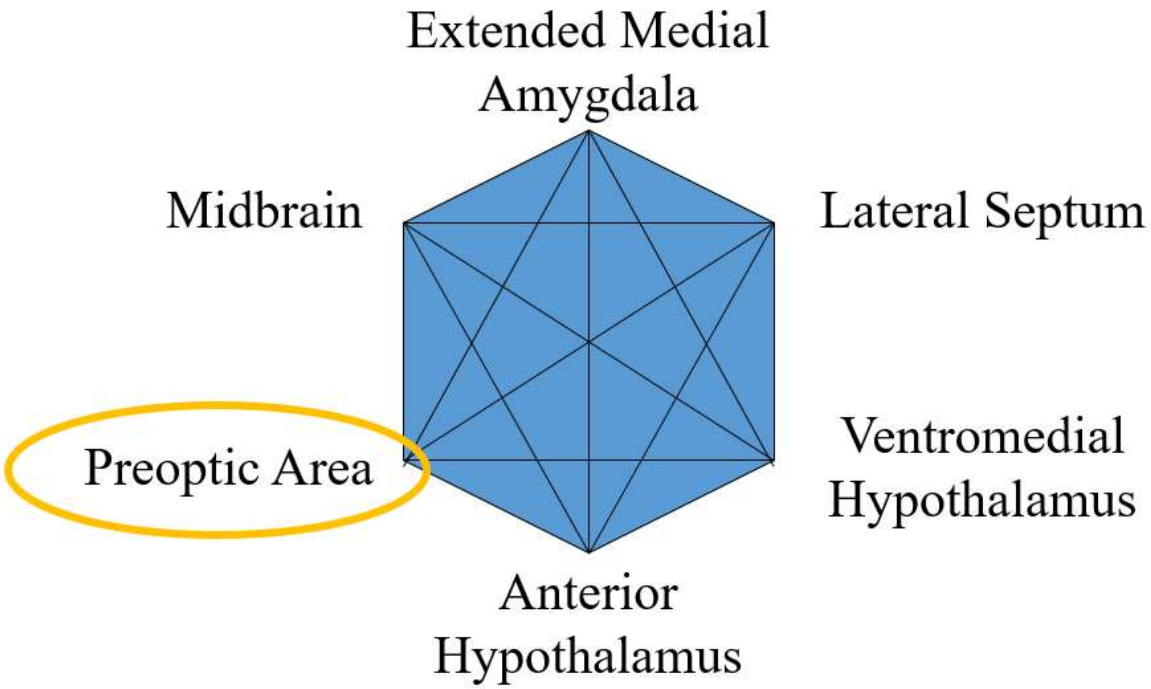

**Figure 3.** Arena used in behavioral trials, including GoPro HERO3 cameras used for recording behavior in the arena (a), video as the variable source of visual information (b), and live anole tethered to a perch (c).

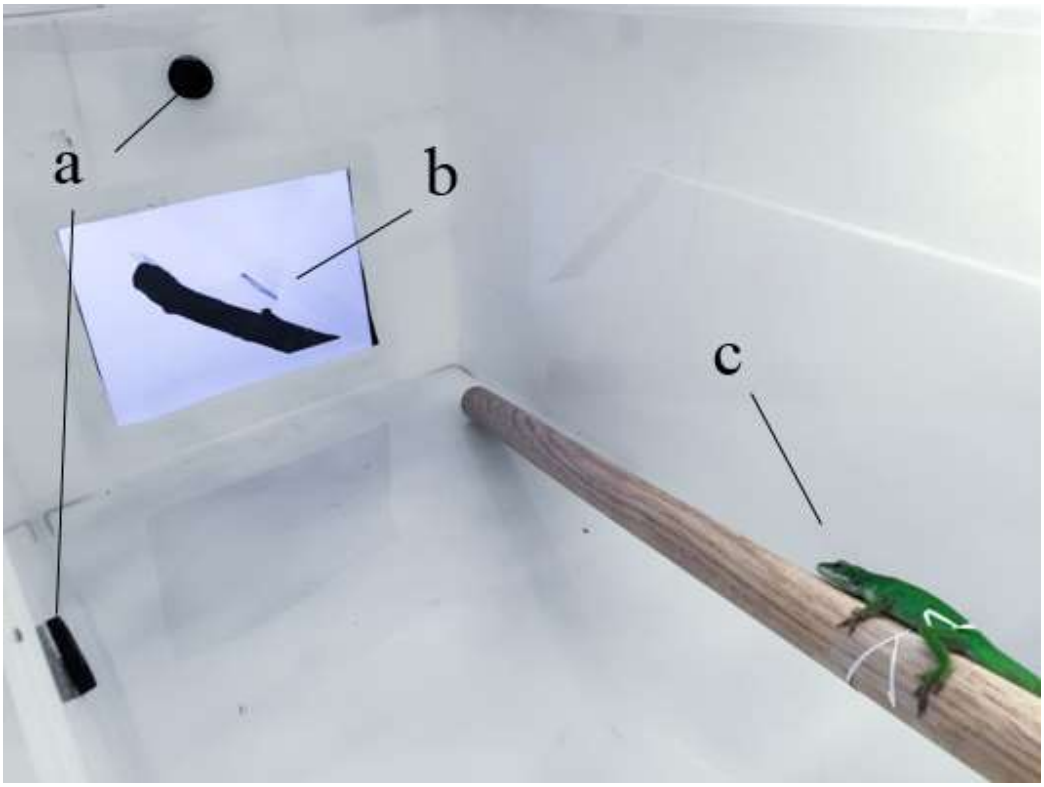

**Figure 4.** Mean lizard attentiveness, as measured by the proportion of time the lizard in each trial spent with their eye tilted towards the screen. Error bars represent +1 standard deviation. Columns with different superscripts are significantly different from one another.

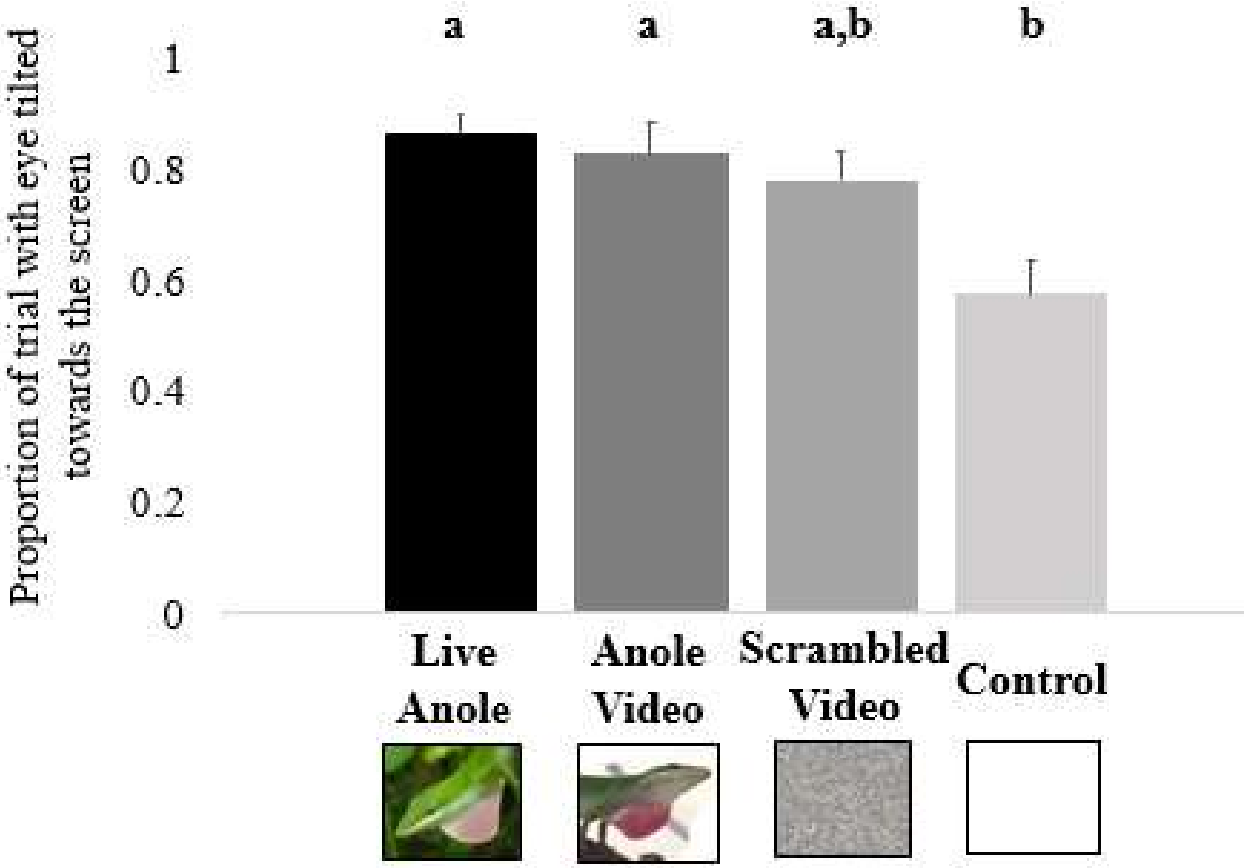

**Figure 5.** Mean number of pushups per trial by treatment condition. Error bars represent +1 standard deviation.

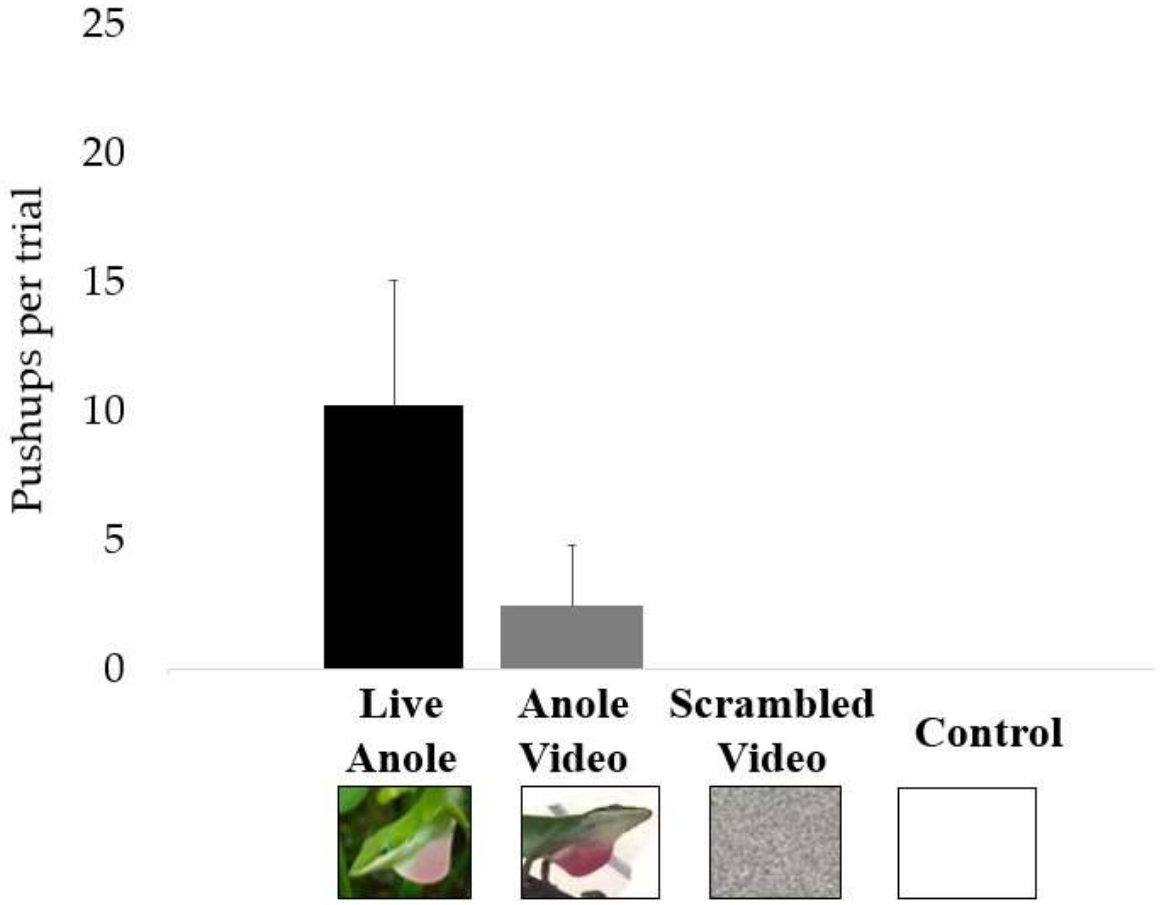

**Figure 6.** Mean number of dewlaps per trial by treatment condition. Error bars represent +1 standard deviation.

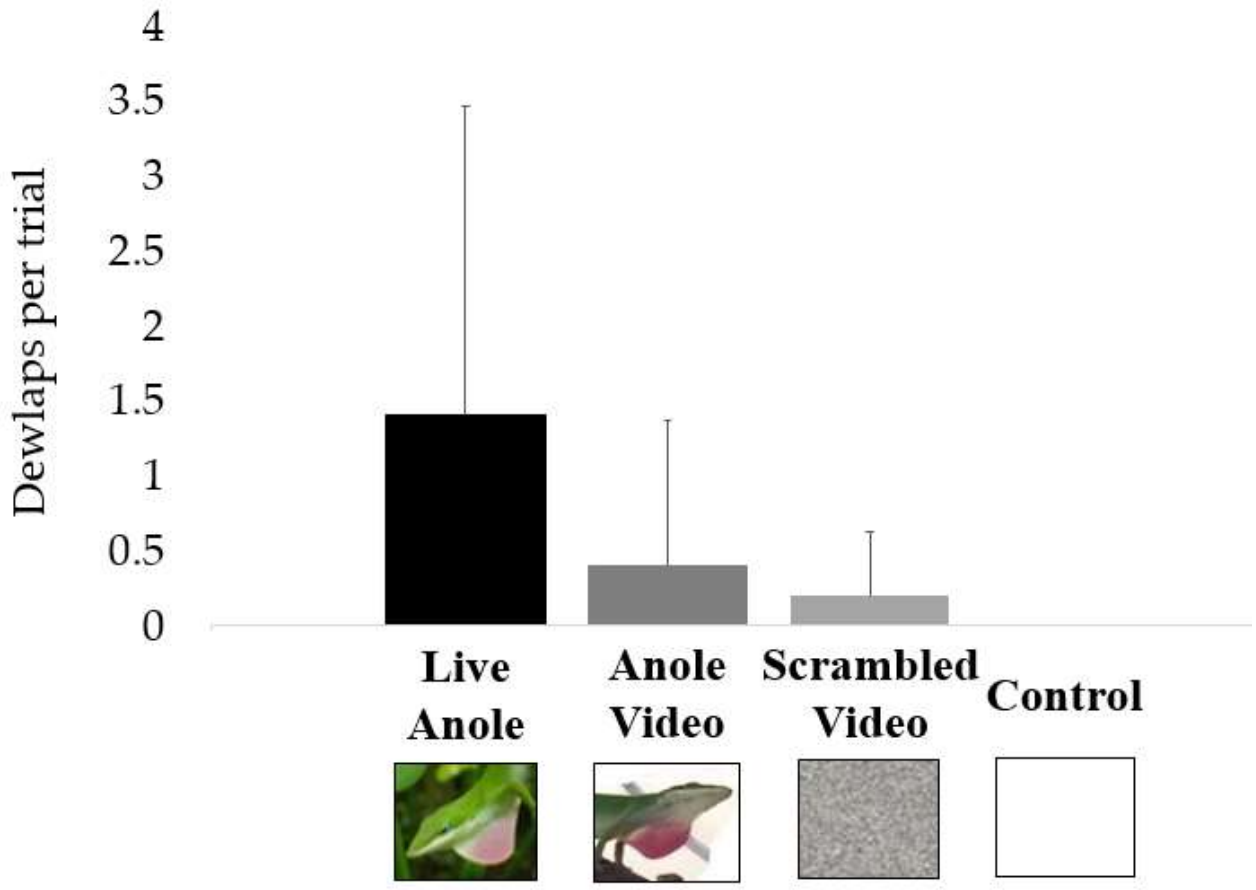

**Figure 7.** Confocal images of the LGN in the green anole brain in the no-primary control, preabsorption control, and an experimental condition. Blue circles indicate DAPI-stained nuclei and red indicates the presence of c-*fos*.

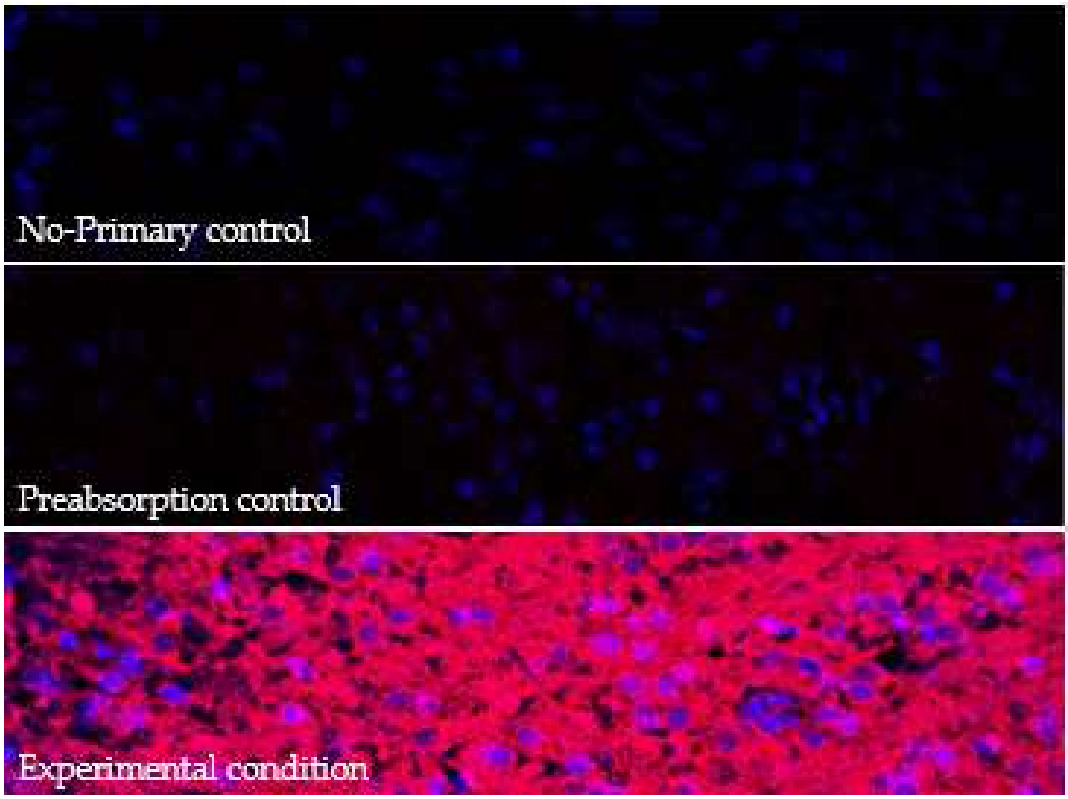

**Figure 8.** Integrated density as a measure of c-*fos* expression in the nuclei of the POA. Error bars represent +1 standard deviation. Columns with different superscripts are significantly different from one another.

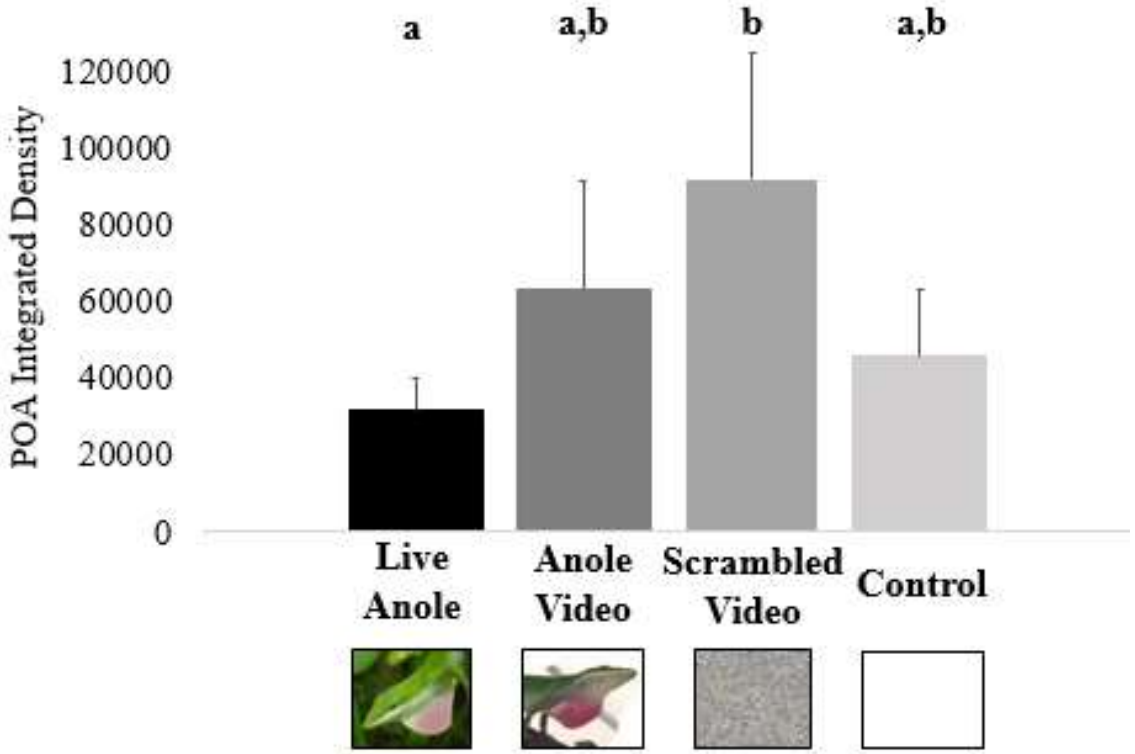

**Figure 9.** Integrated density as a measure of c-*fos* expression in the nuclei of the NROT. Error bars represent +1 standard deviation.

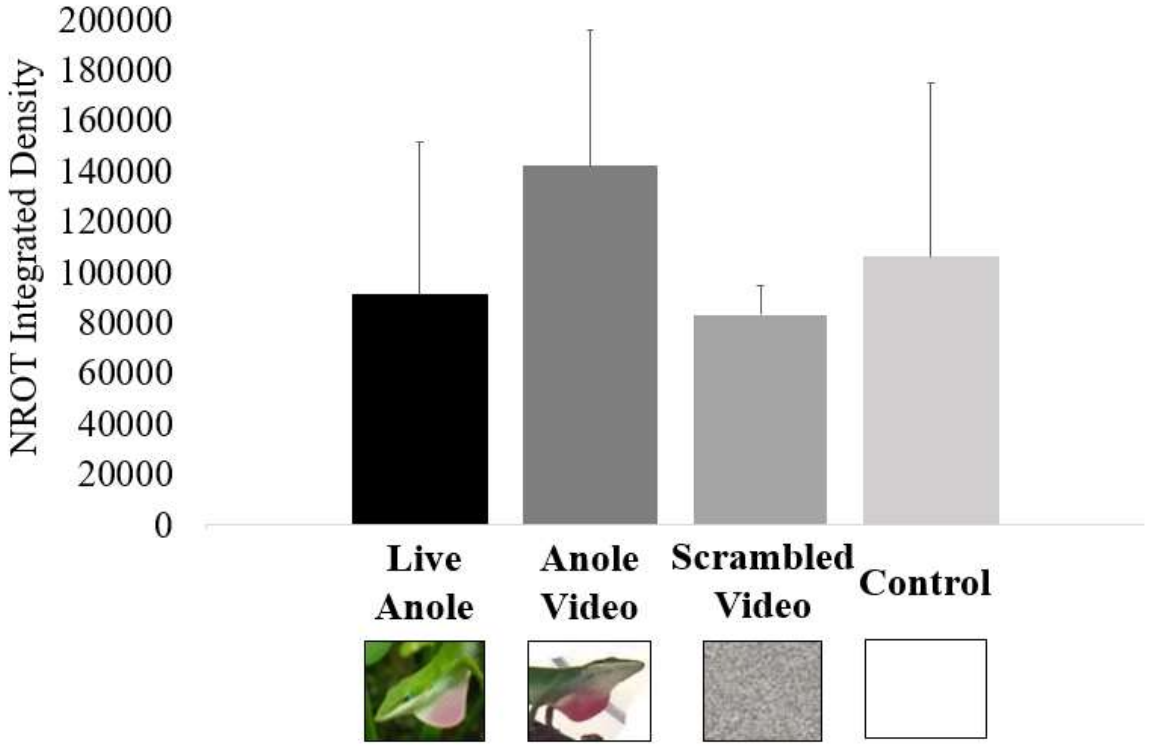

**Figure 10.** Integrated density as a measure of c-*fos* expression in the nuclei of the LGN. Error bars represent +1 standard deviation.

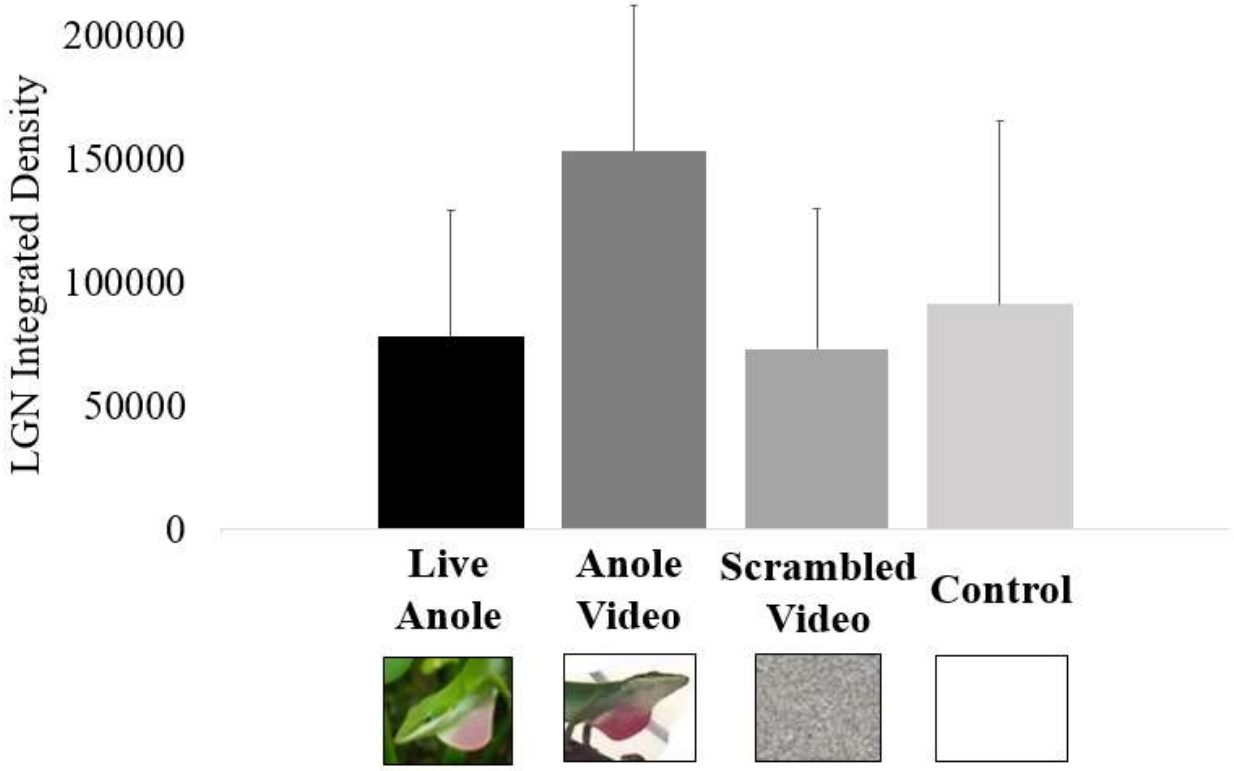

**Figure 11.** Across all conditions (n=15), c-*fos* expression in the LGN is strongly correlated with c-*fos* expression in the NROT.

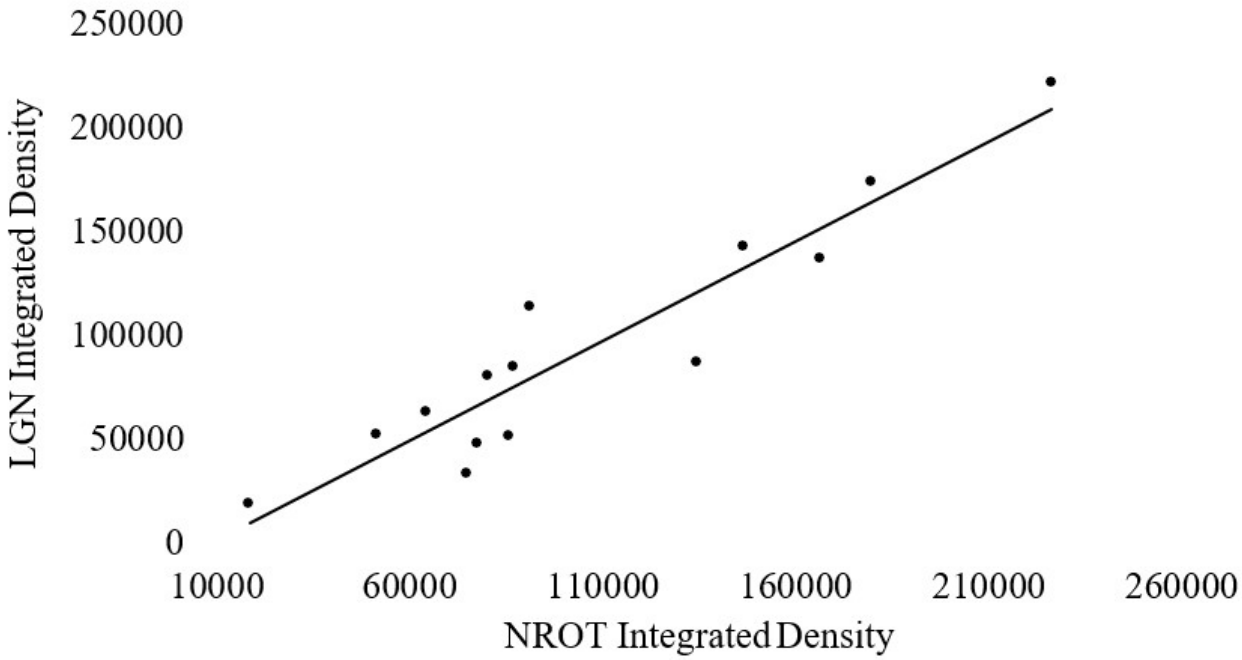

**Figure 12.** Within the Live Anole condition (n=4), c-*fos* expression in the POA is marginally negatively correlated with attentiveness.