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April 13, 2021

The Role of Oxytocin Receptors in Song Learning in Zebra Finches

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## Abstract

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In humans, speech development is a highly social process that requires attention towards and motivation to engage with caregivers. Zebra finches (*Taeniopygia guttata*) share this necessity of social engagement when learning song from adult male tutors. The oxytocin system has been shown to play a central role in social behaviors across species, yet it is unknown whether this system is involved in the attentional and motivational processes that support vocal learning. We tested whether manipulating oxytocin receptor availability during exposure to tutors would impact learning from those tutors. We found a nearly significant trend (of large effect) for juveniles to copy the song of a tutor that was heard during saline (control) treatment more accurately than the song of the tutor that was heard during oxytocin receptor antagonist (OVTA) treatment. We also found that OVTA treatment significantly altered behavior during tutoring sessions. For example, OVTA treatment reduced the frequency with which juveniles engaged in beak swipes at the cage wall closest to the tutor, a behavior that could indicate approach behavior. OVTA also reduced the time the juvenile spent preening, a behavior that, when it occurs during tutoring, had previously been shown to be associated with song learning. Interestingly, we found that tutors sang significantly less to OVTA-treated juveniles, suggesting that tutors are sensitive to OVTA-dependent changes in juveniles. Together these results suggest that oxytocin receptors play a role in socially-guided vocal learning in juvenile zebra finches, perhaps through effects on attention and motivation during tutoring.

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## Introduction

Attention to social cues is a critical component of human learning. As we begin to develop and learn during infancy, social attention helps us acquire new skills. Word learning, for example is positively correlated with the duration of time an infant pays attention to a teacher and with the social engagement of familiar adults (Shneidman, 2009; Yu & Smith, 2012). When we begin to utilize what we have learned, visual cues such as encouraging and enthusiastic facial expressions increase our performance on cognitive tasks by better motivating us (Kohls et al., 2009). Recently, it has been hypothesized that social attention plays an important role in learning speech, as studies have found gaze following, the desire to imitate a caregiver, and joint attention between a child and a caregiver to predict language acquisition (Kuhl, 2007; Brooks & Meltzoff, 2005; Tomasello & Farrar, 1986; Tomasello & Rakoczy, 2003).

While it is clear from these studies that social attention is intricately involved in our learning, it is not the case that social attention manifests the same way among all individuals. Children diagnosed with Autism Spectrum Disorder (ASD) are marked by alterations in vocal communication, attention, and cognitive empathy (Bottini, 2018). The early-emerging social attention processes observed in neurotypical infants were found to be compromised in 2-year-olds later diagnosed with autism (Jones & Klin, 2013). These differences between neurotypical and autistic infants may be due to a difference in what is being attended to. When shown videos of human faces, neurotypical children tend to focus on the individuals' eyes, whereas autistic children focus more on their mouths (Jones et al., 2008). This shift in attention may play a role in delayed vocal development in autistic children compared with neurotypical children (Chericoni et al., 2016). To understand how these differences arise, we must discover the mechanisms that underlie social attention.



To be able to study and manipulate the neurochemical mechanisms that potentially underlie social attention, an animal model must be used. Previous studies have used animal models to uncover the neural pathways by which social attention facilitates pair-bonding and other kinds of relationships with conspecifics. Prairie voles, which are similar to humans in that they are monogamous and biparental, have served as a helpful model for understanding the neurochemistry that drives affiliative behavior in response to some non-mate group members but aggression towards others (Kelly et al., 2018; Lee et al., 2019). Similarly, rats have been helpful in showing how administration of hormones, neuropeptides, or antipsychotics can influence social attention towards new and familiar conspecifics (Thor et al., 1981; Spruijt, 1992; Taylor et al., 2012). Both prairie voles and rats are adequate for studying social attention because, like humans, their peer relationships involve a social component. However, these species do not show vocal learning, and therefore cannot be used to model the role of social attention in the learning of speech. To understand the mechanisms underlying social attention during vocal development, we must choose an animal model with vocal learning that occurs early in life.

An animal that represents an excellent model for understanding social attention during human childhood is the zebra finch (*Taeniopygia guttata*). A unique advantage to using songbirds than other model organisms is that they share our necessity of social engagement for vocal development. Like other songbirds, zebra finches learn their song by selecting, listening to, and engaging with an adult male tutor during their early stages of life. This process is highly reliant on social interaction. When juvenile zebra finches are taught only through audio recordings of adult conspecifics, their songs are poorly learned (Immelmann, 1969). To be able to copy the complex, particular song of an adult conspecific, juvenile zebra finches require interaction with a live tutor (Eales, 1989). The success of their song copying is greatly influenced

by the degree to which their tutors interact with them (Immelmann, 1969). Like juvenile zebra finches, human infants rely on social interaction for the development of their own speech (Goldstein et al., 2003). Thus, zebra finches can be used as a model to understand the neural mechanisms that modulate attention to social cues and thus facilitate vocal learning.

A potential candidate mechanism that may modulate attention to social cues is the oxytocin system (Neumann & van den Burg, 2013). Across species, oxytocin-family peptides have been shown to play a role in many social and reproductive behaviors. Oxytocin-family peptides are signaling molecules that are nine amino acids long and produced mainly by cells in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus (Choleris et al., 2013; Laycock, 2009). Receptors for oxytocin are generally found throughout the brain, although their neural distribution has been found to be highly variable depending on the species (Neumann & van den Burg, 2013). Variation in receptor expression has been shown to be related to pair bonding, responses to stress, socio-sexual behaviors, and species-typical group density (Bales et al., 2008; McCarthy et al., 1997; Bales et al., 2004; Bales & Carter, 2003; Yoshida et al., 2009). “Knockout” mice, which are lacking oxytocin receptors throughout the brain show impairments in social learning that are thought to be mediated by effects on social memory and attention towards familiar conspecifics (Ferguson et al., 2000). In humans, intranasal administration of OT in adults has been shown to positively affect eye gaze towards socially relevant information, emotion recognition, and trust (Guastella et al., 2007; Schulze et al., 2011; Lischke et al., 2001; Kosfeld et al., 2005). These findings suggest that oxytocin may play a role in attention to social cues and pro-sociality.

The hypothesized role of oxytocin in prosocial behavior has led researchers to investigate whether oxytocin administration may aid social attention in children with ASD (Kanat et al.,

2017). This hypothesis is based on a body of evidence showing that, during sensitive periods in early life, oxytocin imposes organizational effects on the brain that can permanently alter its neural structure (Baran, 2017; Boer, 1985; Noonan et al., 1989; Hammock, 2014; Miller & Caldwell, 2015). Research in mice and prairie voles has shown that these effects can impact social behavior. Administering oxytocin in neonatal prairie voles has been shown to cause long-term changes, such as an increase in behaviors indicative of partner preference once they become adults (Bales & Carter, 2003). Blocking oxytocin receptors and other related receptors has also been shown to impact social attachment. When injected with antagonists that blocked oxytocin receptors, juvenile rodents were less motivated to interact with their caregivers and other pups (Ross & Young, 2009; Kojima & Alberts, 2011). Therefore, these receptors may have some influence on the attention given to a social stimulus through their impacts on affiliation.

In zebra finches, oxytocin receptors have been found to be abundant in brain regions important for affiliative social behaviors such as flocking and pair bond formation (Leung et al., 2009; 2011; Goodson et al., 2009). Administration of oxytocin receptor antagonist to zebra finches by peripheral injections significantly reduced gregariousness, approach behaviors towards familiar conspecifics, pair formation, and courtship (Goodson et al., 2009; Pedersen & Tomaszycski, 2012). In juveniles, oxytocin receptors are already expressed in regions important for song learning, such as the auditory forebrain and nuclei that control singing (Davis et al., 2019). Since vocal development in zebra finches is highly reliant on attention towards an adult conspecific, we hypothesized that oxytocin receptor signaling may act to direct attention to song tutors early during song learning, thus facilitating learning of that tutor's song.

In this study, we manipulated oxytocin receptor availability in young zebra finches during the choice of a tutor early during song learning. We treated juvenile males that were naïve

to song with a peripheral subcutaneous injection of either saline solution or an oxytocin receptor antagonist, then exposed them to one of two adult male tutors. Every juvenile experienced both the saline and the antagonist conditions and each tutor was specific to one of the two conditions (within-subjects, cross-sectional design). Thus, juveniles were exposed to only one tutor at a time. Because we hypothesized that oxytocin receptors mediate social attention, we predicted that juveniles would choose to learn the song of the tutor paired with saline instead of the tutor paired with oxytocin receptor antagonist. To quantify learning, we recorded the final, crystallized song of each juvenile after it reached adulthood and compared that song to the song of each of the two tutors.

Our outcome measure of learning, the juvenile's final adult song, was measured well after tutoring sessions had ended. In addition to effects on the final song, we also tested for behavioral effects of the antagonist during the tutoring sessions, well before song develops. Social attention in zebra finches has been tied to specific behaviors that are known to predict the quality of learning (ten Cate, 1986; Hultsch, 1993; Adret, 1993; Houx & ten Cate, 1998). During tutoring, the amount the juvenile preens, which is when it cleans its feathers, positively predicts the quality of song learning (Chen et al., 2016, Houx et al., 2000). Behaviors associated with approach, such as proximity to the tutor, also predict learning (Houx et al., 2000). Other behaviors, such as juvenile vocalization and the general activity of the juvenile seem to be negatively correlated with social attention and affiliation, demonstrating that a juvenile may be more likely to be attending to its tutor when it is quiet and still (Chen et al., 2016; Houx et al., 2000). We therefore quantified a variety of behaviors to test whether the antagonist affected attention to the tutors in an observable way.

## Methods

### *Animals*

Nine male juvenile zebra finches, hereafter referred to as “pupils,” were produced by a breeding colony housed at Emory University and hatched in 2018-2019. All birds were kept in 14x15x17 inch cages and exposed to a 12-hour-light/12-hour dark cycle. Cages were kept in sound-attenuated chambers (21.5x16.24x22.5 inch, Colbourn Instruments, H10- 24TA) to block acoustic interference from the outside environment and prevent unwanted exposure to songs. Birds were given water, seed, cuttlebone, and greens *ad libitum*. To prevent pupils from being exposed to adult song tutors outside of the experimental paradigm, which would contaminate their learning, fathers were removed from the cage when the pupils were four days old. Thus, the pupils were reared only by their female parents, who do not sing (Zann, 1996).

To ensure that all the pupils we selected were male and, therefore, could learn song, we used a Polymerase Chain Reaction (PCR) from DNA extracted from blood samples— a sex-determination method established by Griffiths et al. (1998). Sex was later confirmed by observing birds’ feather patterns. All of our procedures were approved by the Emory University Institutional Animal Care and Use Committee.

### *Recording Song*

The following methods for recording zebra finch song were conducted as described by Rodríguez-Saltos (2019). We obtained recordings of song from the pupils’ fathers and the experimental tutors prior to the study, and from the pupils after they reached adulthood at the end of the study. All songs were recorded in a sound-attenuated booth using a TASCAM DR-7MKII recorder. In some cases, a female or a juvenile was presented to the adult male in order to stimulate singing. Recordings were then cleaned in Audacity (2.3.2), an audio editing and

recording application, using its “Background Noise Reduction” tool. Call-like introductory notes, typical in zebra finch songs (Price, 1979), were removed from the recordings. For the songs used for playback (see *Tutoring*, below), audio was smoothed using the application’s “Fade In” and “Fade Out” tools.

### *Selection of Tutors*

Adult male tutors were selected on the basis of the frequency of their singing, which was determined by placing cages of adult males next to cages of either pupils or females for one hour and noting which males produced the most vocalizations. To limit potential bias towards learning one of the two tutor songs, each pupil was matched to tutors with songs dissimilar to that of the pupil’s father. This dissimilarity was assessed by visually inspecting spectrographs created in Audacity. We looked for shared “syllables,” or short, stereotyped sounds that are separated by a brief period of silence (Fig. 1; Zann, 1996). We matched each pupil to tutors that shared no more than two syllables with the pupil’s father (Range 0-2,  $M = 0.667$ ,  $SD = 0.623$ ). Moreover, for each pupil, the OVTA and Saline tutors shared no more than two syllables with each other (Range, 0-2,  $M = 0.6$ ,  $SD = 0.8$ ). The shared syllables were primarily stereotyped, call-like sounds that typically vary very little among individuals (Zann, 1996).

### *Tutoring*

All tutoring trials took place when the pupils were between 27 and 42 days-post-hatch (dph), which corresponds to the peak period of song learning from a tutor (Böhner, 1990). Thirty minutes before each of four tutoring trials, pupils were treated with either saline or an oxytocin receptor antagonist (OVTA) [d(CH<sub>2</sub>)<sup>5</sup>1, Tyr(Me)<sup>2</sup>, Thr<sup>4</sup>, Orn<sup>8</sup>, des-Gly-NH<sup>29</sup>] -Vasotocin trifluoroacetate salt (Bachem), dissolved in saline. Both solutions were administered via a 0.05 mL subcutaneous injection into the shoulder area between the feather tracts. Trials were

separated by two days and the treatments, OVTA or saline, were alternated. The order in which the pupils received each treatment was counterbalanced, such that some pupils received OVTA first and vice versa.

For each pupil, each of the two treatments was always associated with the same tutor; in other words, there was an “OVTA tutor” and a “Saline tutor” for each pupil. Pupils spent two tutoring sessions with each of their tutors, for a total of four sessions per pupil. Each tutoring session lasted 50 to 60 minutes. First, a speaker was placed into a walk-in sound-attenuating booth. The speaker was set to project sound at 75 dB. Then, immediately after receiving the subcutaneous injection of OVTA or saline, a pupil was placed into a testing cage, brought into the booth, and given 30 minutes alone to acclimate to its new environment before the tutoring session commenced. The tutor was given about 15 minutes to habituate in a separate, identical booth. When brought into the booth with the pupil, the tutor was placed such that its cage was positioned in between the speaker and the pupil’s cage. We defined the start of the tutoring session as the moment when the experimenter left the room after placing the tutor in the booth.

We took steps to control the number of times each pupil heard each tutor’s song during tutoring sessions, such that each pupil heard roughly the same number of songs from each of the two tutors. On the basis of our estimation of each tutor’s singing rate, which was made when selecting the tutors, we arrived at a predetermined number of songs that each pupil should hear, which was equal for each of the two tutors. If this target number was not reached by 45 minutes into the session, recordings of that tutor’s song were played from the speaker during the last fifteen minutes of the tutoring session. In instances when the tutor sang at a rate higher than anticipated, tutoring sessions were cut a few minutes short in order to control the number of

songs heard by the pupil. The average session duration was 59 minutes  $\pm$  5 seconds. After each session, the pupil and the tutor were brought back to their respective home cages.

After all four tutoring sessions were completed, as part of another study, all of the pupils were housed singly in sound-attenuating chambers where they were allowed to self-tutor using keys that played the songs of their saline and OVTA tutors. Exposure to the two songs was balanced such that the pupils heard the same number (30) of each song per day. Pupils were housed with the keys until their final adult song was recorded.

### *Behavioral Observation and Quantification*

We filmed the tutoring sessions using a video camera placed in the sound-attenuating booth. After all the trials were completed, the recordings were observed using Behavior Observation Research Interactive Software (BORIS, v.7.9.19). We labeled the behaviors we frequently observed pupils performing during the tutoring sessions. When selecting which of these behaviors to analyze, we chose behaviors that others have previously found to be predictive of song learning: preening and approach, which have been found to be positively associated with learning; activity, and vocalizations, which have been reported to be negatively associated with learning (Houx et al., 2000; Chen et al., 2016). Preening is a grooming behavior that we defined as when the pupil cleaned either its feathers or another part of its body. To quantify approach, we used three behaviors. First, we quantified the total time (percentage of time during the session) that the pupil spent in the section closest to the tutor, pre-labeled as the 12-centimeter area of the pupil's cage located closest to the tutor's cage. This area of the cage was designated as "t1." Our second measure of approach was "close swipe," which was counted each time the pupil approached the wall of its own cage closest to the tutor and touched its beak against that wall.



To quantify activity level, we chose a behavior that occurred frequently in the most active pupils: two-foot wall contacts, in other words when the pupil placed both of its feet on any side of the cage. This behavior indicates that the bird is flying around inside the cage, and is thus quite active. We also quantified beak wipes, defined as when the pupil either rubbed or pecked its beak at least twice on its perch, irrespective of its location in the cage. Finally, we counted all vocalizations made by the pupils. These vocalizations did not include song, as the tutoring sessions took place largely before birds are entering the subsong phase (Immelmann, 1969).

In addition to the pupil's behavior we also scored tutor song. We defined a tutor song bout as an instance of continuous singing with at least a one-second gap between bouts. The duration of zebra finch song is highly variable, with a range of two to 12 syllables (Bruno, 2017). Syllables may also repeat more than once during a single song iteration (Tchernichovski et al., 2001). Because we were interested in song rate more than the total duration of singing, we scored a song bout as a point event. Song bouts were the only behaviors we recorded for the tutor; all other behaviors were scored only for the pupil.

Each occurrence of the behaviors above was marked while observing the trials through BORIS. While some behaviors happened instantaneously, others lasted over a duration of time. We labeled these behaviors as point events and state events, respectively. For point events (close swipe, beak wipe, two-foot wall contacts, pupil vocalizations, and tutor song bouts), we calculated the total number of instances of a behavior. The number of instances was then divided by the total number of minutes in the trial, to arrive at rate per minute. For state events (preening and time spent in t1), we calculated the sum of each duration of a behavior in seconds. These durations were then divided by the total number of seconds in each trial to arrive at a proportion of each trial engaged in the behavior.

### *Song Segmentation and Spectral Analysis*

When the pupils reached adulthood, their songs were recorded and cleaned in a fashion similar to the tutors' songs (see above, *Selection of Tutors*). Two of the pupils became ill and were excluded from the study before reaching adulthood. A third pupil did not self-tutor using the keys, so it was also excluded, bringing our sample size for the song analysis to six birds. Songs were recorded at 101-103 dph, the point at which pupils have completed their vocal development and produced a stereotyped song that does not change much thereafter (Price 1979). Song recordings were imported as .WAV files into Sound Analysis Pro (SAP). SAP generates sound spectrograms allowing visualization of a song's acoustic features such as its amplitude, entropy, and pitch (Tchernichovski et al., 2000). To calculate similarity between tutor and pupil songs, we segmented songs into syllables on the basis of an amplitude threshold (Tchernichovski et al., 2000; Fig. 1).

Thresholds were adjusted for every song because each one was sung at a different distance from the microphone; thus, song-specific amplitude thresholds allowed for each song to be properly segmented. Similarity scores were calculated using SAP's "Song Similarity" tool, which compares the acoustic features of one song's syllables to another and quantifies similarity (Tchernichovski et al., 2000). Similarity scores were calculated for each pupil-tutor pair using three to six song exemplars (which represented all of the available song recordings) for each pupil. We used the maximum similarity score for each pupil-tutor pairing for the analysis (Shi et al., 2017; 2018).

### *Statistical Analysis*

To test for effects of the OVTA on behavior and song learning, we used linear mixed models (LMMs) in R (v4.04). Separate tests were conducted for each measure. In all models,

Subject ID was included as a random effect to accommodate the within-subjects design. For all variables except song similarity, we used the following model:  $\sim \text{Trial} + \text{Treatment} + (1 \mid \text{Subject ID})$ , which allowed us to control for the effects of Trial (i.e. the first or second trial for each treatment). To perform the LMM analyses, we used the *lmer* function in the lme4 package (Bates et al., 2014). To test for a significant effect of Treatment on each variable of interest, we performed a likelihood ratio test using the *anova* function to compare the full model to a reduced model with Treatment removed. As a measure of effect size, we calculated Cohen's  $d_{av}$  as described by Cumming (2012; see also Lakens, 2013), which takes into account the within-subject design.

## Results

The effects of OVTA treatment on all of the behaviors we measured are shown in Figures 2 and 3. Figure 2 shows the data for each individual bird; Figure 3 shows the means for each measure. We found that juveniles spent proportionally less time preening during the OVTA condition than during the saline condition (Saline:  $M = 2.964 \pm 2.537 \text{ SD}$ , OVTA:  $M = 1.606 \pm 1.514 \text{ SD}$ ) ( $X^2(1) = 9.644$ ,  $p = 0.002$ ,  $d_{av} = 0.64$ ). While we did not find a difference between conditions in the percentage of time spent in the third of the cage closest to the tutor during trials (Saline:  $M = 69.361 \pm 28.663 \text{ SD}$ , OVTA:  $M = 79.929 \pm 27.247 \text{ SD}$ ) ( $X^2(1) = 1.680$ ,  $p = 0.195$ ,  $d_{av} = 0.42$ ), we did find that juveniles swiped less at the side of the cage closest to the tutor during the OVTA condition. (Saline:  $M = 0.101 \pm 0.115 \text{ SD}$ , OVTA:  $M = 0.0337 \pm 0.0472 \text{ SD}$ ) ( $X^2(1) = 5.0218$ ,  $p = 0.025$ ,  $d_{av} = 0.81$ ). We did not find any difference between conditions in activity, defined as the frequency of two-foot contacts with cage walls, between the saline and OVTA conditions (Saline:  $M = 0.216 \pm 0.613 \text{ SD}$ , OVTA:  $M = 0.193 \pm 0.462 \text{ SD}$ ) ( $X^2(1) =$

0.0234,  $p = 0.878$ ,  $d_{av} = 0.11$ ), or juvenile vocalizations (Saline:  $M = 0.513 \pm 0.762$  *SD*, OVTA:  $M = 1.223 \pm 3.056$  *SD*) ( $X^2(1) = 0.710$ ,  $p = 0.400$ ,  $d_{av} = 0.35$ ). However, the number of song bouts sung per minute by the OVTA tutors was significantly less than that sung by the Saline tutors (Saline:  $M = 0.636 \pm 0.639$  *SD*, OVTA:  $M = 0.318 \pm 0.340$  *SD*) ( $X^2(1) = 4.355$ ,  $p = 0.0367$ ,  $d_{av} = 0.71$ ). We did not find any difference in juvenile beak wiping between the two conditions (Saline:  $M = 0.0701 \pm 0.111$  *SD*, OVTA:  $M = 0.0387 \pm 0.0352$  *SD*) ( $X^2(1) = 1.223$ ,  $p = 0.269$ ,  $d_{av} = 0.41$ ). Finally, although the effect of OVTA treatment on maximum song similarity was not significant, there was a compelling trend for the OVTA to reduce learning; the size of this effect was large (Saline:  $M = 83.5 \pm 11.726$  *SD*, OVTA:  $M = 63.167 \pm 23.617$  *SD*) ( $X^2(1) = 3.662$ ,  $p = 0.056$ ,  $d_{av} = 1.15$ ).

## Discussion

In this study, we tested the role of oxytocin receptors in early vocal learning. In zebra finches, oxytocin receptors are abundant in brain regions important for song learning (Davis et al., 2019). We determined pupils' song learning outcome after they had been exposed to an OVTA-paired tutor and a saline-paired tutor to observe whether treatment condition impacted song copying. Previously, blocking oxytocin receptors in zebra finches was shown to inhibit social behaviors such as approach and pair bonding (Goodson et al., 2009; Pederson & Tomaszycki, 2012). As song learning depends on social interactions (Immelmann, 1969; Eales, 1989), we also tested whether blocking oxytocin receptors affected behavior during tutor-pupil interactions.

Our data suggest that oxytocin receptor antagonism affected pupils' song learning outcomes (Fig. 3H). Similarity scores were lower between pupils and OVTA-paired tutors than

between pupils and Saline-paired tutors. Although the effect was not statistically significant at  $p = 0.056$ , the effect size was large. This finding implicates oxytocin receptors in vocal development. However, we can only speculate about how these receptors are impacting this process. Chen et al. (2016) suggested that the efficiency of tutoring is highly contingent on attention and vice versa: when tutors directed their songs towards their pupils, pupils paid more attention and, consequently, copied these tutors' songs better than pupils that were inattentive. One possibility is that when blocking oxytocin receptors, we are obstructing a pupil's natural response to attend to a tutor which, in turn, inhibits song copying. This possibility would be in line with research in humans showing that attentiveness predicts early learning (Shneidman, 2009; Tomasello & Farrar, 1986). Interestingly, our findings show that oxytocin receptor antagonism impacted not only song learning outcomes but also behaviors during tutoring sessions. These behavioral differences may be indicating an effect of oxytocin antagonism on attention. Oxytocin receptors may also play a role in a multitude of factors that could facilitate song learning, such as a pupil's level of motivation to learn song, its ability to experience social stimuli as rewarding, and its level of anxiety. Any of these effects could produce changes in behavior during tutoring sessions.

Of the behaviors that were scored in this study, the one most affected by OVTA treatment was preening (Fig. 3A). Preening occurs mostly while birds are stationary, meaning not actively flying or hopping around (Delius, 1988). Considering that stillness during tutoring is thought to be associated with attention (Houx et al., 2000), preening behavior may occur more frequently when a bird's attention is directed toward a song tutor. Preening behavior and stillness during tutoring were previously shown to correlate positively with the degree of song copying (Houx et al., 2000; Chen et al., 2016). This finding may be the result of stronger song copiers having a

greater desire to listen to their tutors. Blocking oxytocin receptors in pupils may inhibit this desire, leading indirectly to a decrease in preening.

We did not see an effect of OVTA on our measure of approach, which was the duration of time a pupil spent in the 12-centimeter area of its cage closest to its tutor's (Fig. 3B). However, we did see that pupils swiped their beaks on the cage wall closest to their tutor's cage less frequently in the OVTA condition (Fig. 3C). Thus, although blocking oxytocin receptor availability did not impact approach itself, it decreased a behavior associated with approach. Behavior directed towards a tutor is thought to demonstrate active listening to tutor song (ten Cate, 1986). As such, researchers have previously regarded approach as a behavior that indicates active listening (Mann & Slater, 1994; Houx & ten Cate, 1998; Houx et al., 2000). Reductions in beak swiping, as it is a behavior related to approach, may be indicating that oxytocin receptor antagonism is decreasing a pupil's desire to engage in active listening.

Our most unexpected finding was that tutor song bouts occurred less frequently in the OVTA condition than in the Saline condition (Fig 3F). Importantly, although we detected a difference in the number of tutor song bouts between conditions, it was not the case that pupils were less exposed to their OVTA-paired tutor's songs than their Saline-paired tutor's songs. Using the speaker placed between both cages prior to tutoring sessions, we ensured that the amount of live song plus the amount of tutor song playback was equal between conditions. Thus, differences in tutor song bout frequency did not cause an imbalance in song exposure. That being said, hearing the tutor sing live may be more salient or rewarding to the pupil than hearing a playback, which could have affected learning. It is also possible that other tutor behaviors, such as tutor activity, which we did not score, or more subtle behaviors indicating engagement with a

pupil occurred at different rates between treatment conditions. The song learning outcome may have been impacted by these potential differences as well.

Our findings for tutor song bouts show that administering oxytocin receptor antagonist to the pupil reduced song behavior in the tutor. It has been previously suggested that pupil behavior may influence tutor singing rate (Adret, 1993; ten Cate; 1994). In our experiment, it could be the case that tutors engaged in less singing behavior due to changes in behavioral cues given by the pupil after the antagonist had been administered. Unfortunately, it is still unclear which behavioral cues may impact tutor song rate (Houx & ten Cate, 1998). Thus, we do not know which behaviors OVTA changed in the pupils that caused the effect seen on their tutors.

Our data suggest that blocking oxytocin receptors has no effect on pupil activity, as neither our direct measure of activity (number of 2 feet contacts) nor the frequency of vocalizations differed between groups (Fig. 3D-E). Because it shows that pupils were just as active when they were administered OVTA, this finding suggests that the antagonist did not make pupils feel ill or lethargic. It is important to note, in light of our finding that OVTA inhibited learning, that other authors have reported a negative relationship between activity and learning (Houx et al., 2000, Chen et al., 2016). Thus, we might have expected to see an increase in activity in the OVTA condition. However, in those previous studies, activity and vocalizations were measured specifically when the tutor was singing. In contrast, in this study, we recorded only the general frequency of a behavior during the entire tutoring session. If we had scored activity occurring contingent with tutor song, as others have done (Houx et al., 2000; Chen et al., 2016), we may have found an effect of oxytocin receptor antagonism on activity. Additionally, we may have seen larger effects on behaviors that did occur less frequently in the OVTA condition.

Another caveat in this study comes from the fact that oxytocin receptor antagonism was administered peripherally, meaning that we had no control over where in the brain the antagonist traveled. We know from past research that oxytocin receptors are expressed in brain regions associated with vocal development as well as in many other cortical, limbic, hypothalamic, and brainstem regions in zebra finches (Davis et al., 2019; Leung et al., 2009; 2011). To determine which receptor populations are responsible for alterations in vocal development in finches and the role they each play, we will need to manipulate these populations independently in future studies through central administration of antagonist. Additionally, although it has been shown previously that peripheral injections of OVTA affect sociality in zebra finches (Goodson et al., 2009), it may be the case that the antagonist did not bind to regions in the central nervous system at all, as it has been suggested that only a small percentage of oxytocin-like molecules successfully cross the blood-brain barrier (Ermisch et al., 1985; Kendrick et al., 1986; Englemann et al., 1996). Instead, the antagonist may be acting on peripheral mechanisms outside of the brain (Cushing & Carter, 1999).

It is important to highlight that administration of OVTA blocks two potential endogenous ligands: mesotocin and vasotocin, which are the homologues of oxytocin and vasopressin, respectively, in birds (Leung et al., 2009; Kelly & Goodson, 2014). Oxytocin receptors in birds have a high affinity for both of these peptides (Voorhuis et al., 1990; Leung et al., 2009). Previous research has shown that administering vasotocin in zebra finch hatchlings significantly increased song copying success; additionally, blocking V1a receptors, which binds vasotocin more exclusively, in hatchlings worsened song copying (Baran et al., 2017). These findings from previous studies suggest that vasotocin impacts the song learning outcome when it binds to V1a. To test whether vasotocin is also the ligand responsible for the effect seen here of oxytocin



receptors on song learning, specifically during tutoring, separate studies must be conducted that treat birds centrally with vasotocin. Still, it may be the case that both mesotocin and vasotocin differentially impact song learning when binding to oxytocin receptors.

In conclusion, we have drawn an apparent link between oxytocin receptors and song learning in juvenile zebra finches, a species in which some mechanisms of vocal development may be shared with humans (Immelmann, 1969; Eales, 1989; Goldstein et al., 2003; Doupe & Kuhl, 1999). The behavioral effects of blocking oxytocin receptors may indicate a change in attention. Steps should be taken to identify the oxytocin receptor populations and the endogenous ligands responsible for these effects. In future research, oxytocin receptors should be blocked centrally, in brain regions important for song learning such as the auditory forebrain, HVC, and the dorsal arcopallium; researchers could also target the lateral septum, which has been linked to social motivation and therefore may also serve a purpose in song learning (Davis et al., 2019). If effects on song learning are seen, the ligands binding to these regions should then be identified. With this additional research, we will have a more complete idea of the role oxytocin receptors play in vocal development.

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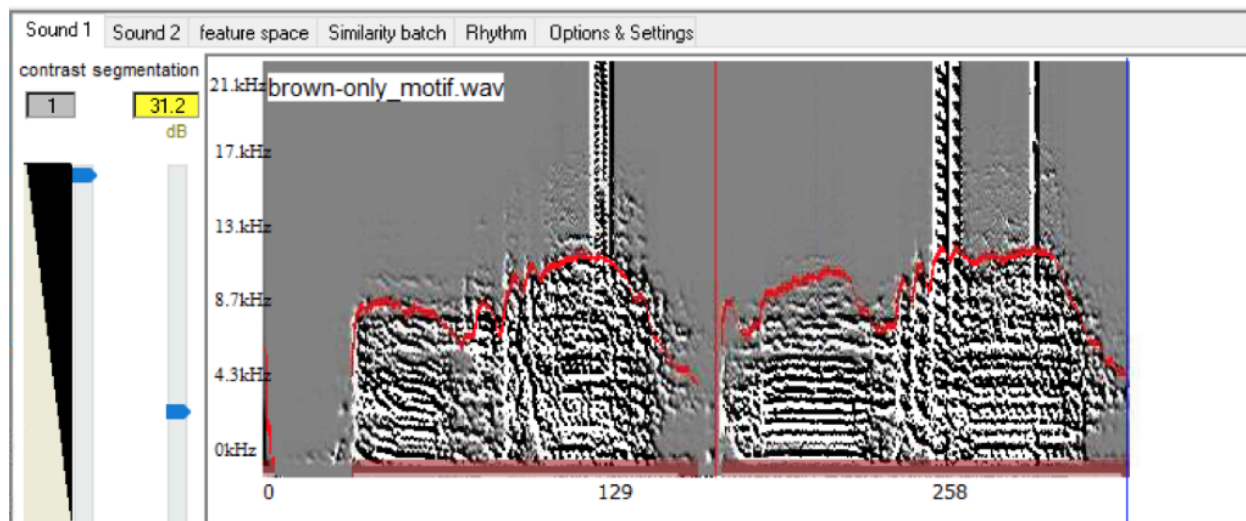
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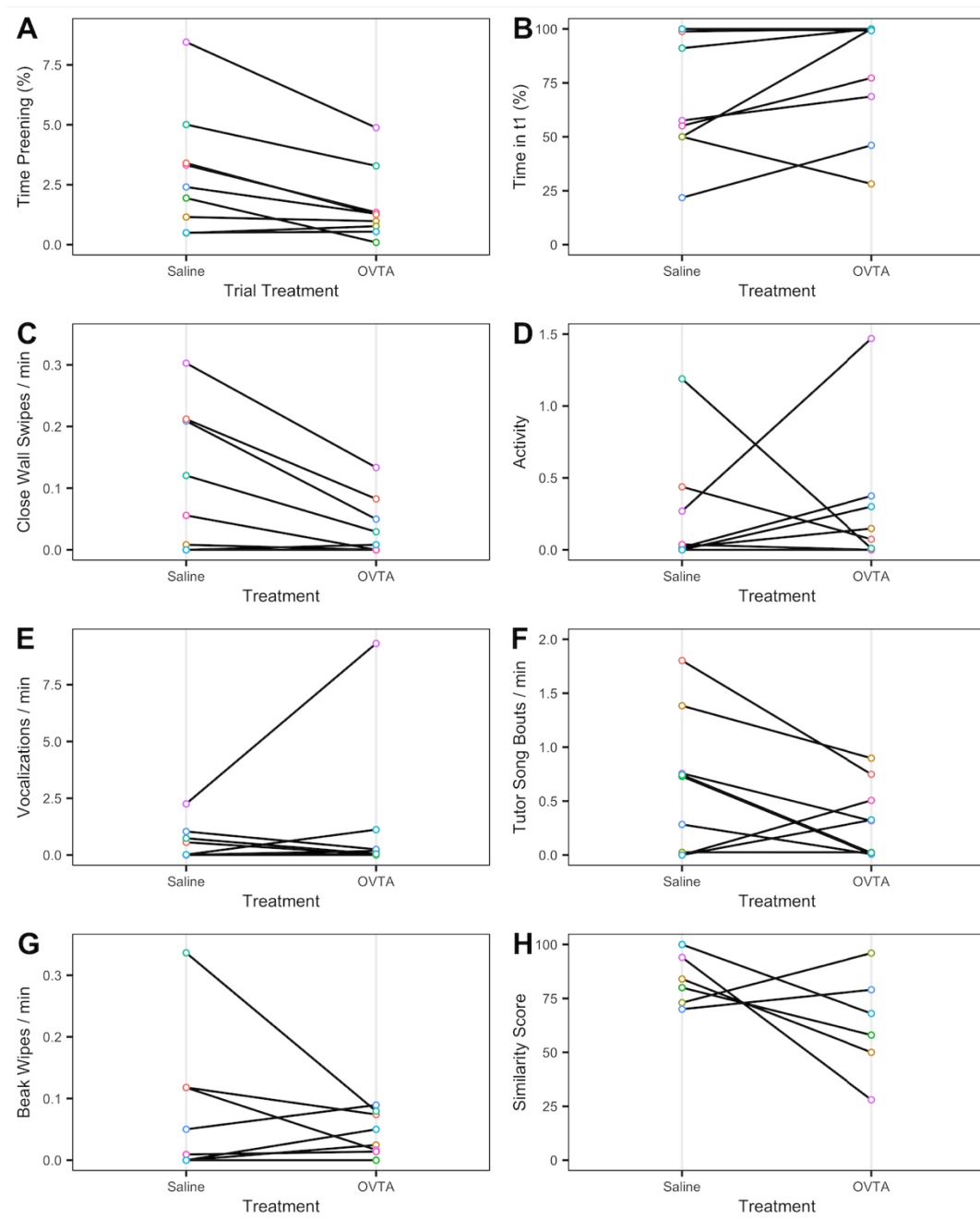
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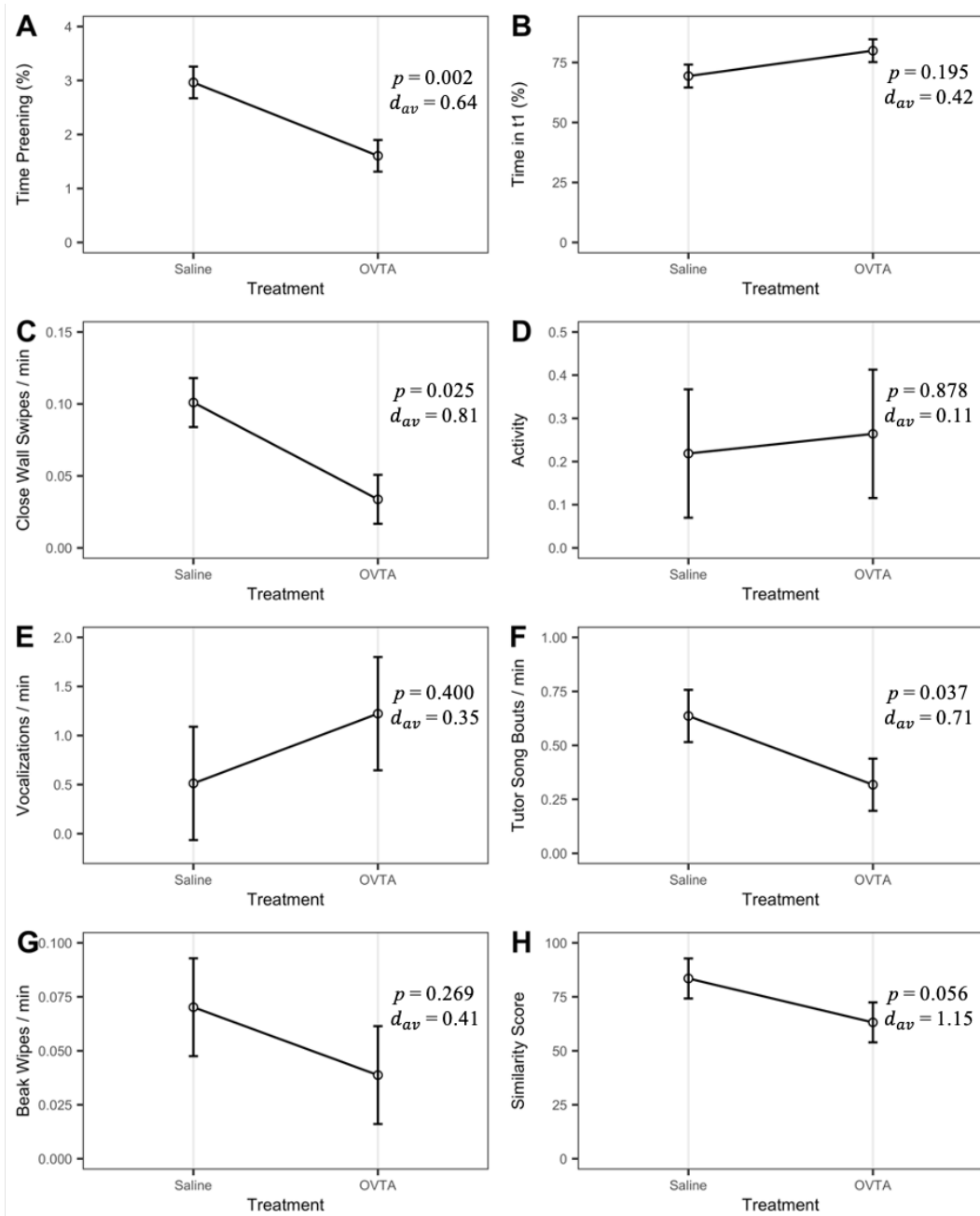
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**Figure 1. Example of a Sound Spectrogram.** A spectrogram is shown in Sound Analysis Pro (Tchernichovski et al., 2000), which plots song duration in milliseconds across the X-axis and frequency in kilohertz (kHz) on the Y-axis. This spectrogram shows multiple harmonics, meaning that the bird's song contains multiple pitches simultaneously. The amplitude threshold for this song can be seen in yellow on the top left. This threshold was used to segment the song into syllables, which are indicated by red bars under the spectrogram. In this example, the song was segmented into two syllables, separated by a gap between the red bars.



**Figure 2. Comparisons of Saline and OVTA conditions for each pupil.** Each line on each plot represents one of the pupils in the sample. The two treatment groups are marked on the x-axis while the pupils' scores are indicated by color-coded points on the y-axis. (A-G): Data for each of the previously defined behaviors (see Methods) was calculated across both treatment conditions and plotted for each bird. The values for activity (sum of two-footed wall contacts), beak wipes, close swipes, pupil vocalizations, and tutor song are shown as the number of instances per minute. For preening and location in t1, the percentage of the trial spent engaging in the behavior is shown. The means across both tutoring trials for each treatment condition are plotted. (H): The maximum similarity score a pupil achieved for each tutor, calculated by comparing the pupil's song to that tutor's song (see Methods), is shown on the y-axis.



**Figure 3. Effects of OVTA treatment on behavior and song learning.** Mean scores for saline and OVTA trials are plotted. To accurately depict the within-subjects variation in the error bars, the between-subjects variation was removed using the *summarySEwithin* function in R (v4.04) which implements the method in Cousineau (2005) with the correction factor in Morey (2008). The standard error of the mean (SEM) is therefore equal between conditions for each score. The p values from linear mixed models (see Methods) are shown. Effect size is Cohen's  $d_{av}$  (Cumming, 2012; Lakens, 2013)