

Running Head: TESTOSTERONE AND VOCAL RECOGNITION

The Effect of Prenatal Testosterone Exposure on Maternal and Sibling Vocal Recognition in

Lambs

by

Belinda Shih

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Advisor: Dr. Theresa Lee

## Abstract

Mutual recognition of siblings and mothers by offspring reinforces bonding and later attachment in life. Early behavioral data suggests that females spend more time with their siblings and mother than do males. To date, there is little research on sex differences in vocal recognition of mothers and siblings by young ungulates. This study hypothesized that auditory cues are sufficient for young lambs to recognize close kin during the nursing period, but males and lambs exposed to prenatal androgens will be less responsive than females. Lambs were used in one or both mother and sibling call playback tests. Results from the study confirmed previous research that auditory cues are sufficient for kin recognition. Sex effects were less clearly differentiated than treatment effects. T-treated animals and controls also showed opposite behavioral responses between the tests, with an overall reduced response in the sibling test. Discussion of these results along with suggestions for future studies is presented.

## The Effect of Prenatal Testosterone Exposure on Maternal and Sibling Vocal Recognition in Lambs

Mutual recognition of mother, offspring, and siblings is frequently established early in life and reinforces relationship bonding (DeCasper & Fifer, 1980). Among humans, this feature facilitates secure attachment as well as social and language development (DeCasper, Lecanuet, Busnel, Granier-Deferre, & Maugeais, 1992; Newberry & Swanson, 2007; Purhonen, Kilpeläinen-Lees, Valkonen-Korhonen, Karhu, & Lehtonen, 2003). Among animals, this feature ensures proper maternal investment and offspring survival (Illman, Schrader, Špinka, & Šustr, 2001). Consequently, establishment of kin identification between mothers and offspring as well as between siblings has strong positive implications in future social and physiological behavior in most mammals. This unique recognition ability between mother and offspring and between siblings, however, may be affected by prenatal androgen exposure, which has been shown to physiologically and behaviorally alter sex-typical behaviors of mammals (Dittman, Kappes, Kappes, Börger, Stegner, Willig, & Wallis, 1990; Manson, 2008; Mathews, Fane, Conway, Brook, & Hines, 2009; Pasterski, Hindmarsh, Geffner, Brook, Brain, & Hines, 2007). While recognition relies on several sensory cues, this study will focus on the significance of auditory cues and the impact of prenatal testosterone on kin identification in ungulates.

The importance of a species' ability to identify maternal and kin relationships is stressed through the species' maternal behavior and offspring development. Mutual recognition between the mother and her offspring is instrumental in establishing maternal behavior among many mammals (Nowak, 1990; Searby & Jouventin, 2003). In highly social species, such as humans and ungulates, maternal care is frequently directed only towards the mother's offspring (Poindron, Lévy, & Keller, 2007). This exclusive care increases the potential of reproductive

success of the mother and also maximizes her fitness (Illman et al., 2001; Trivers, 1972).

Mother and young recognition is involved with the development of maternal care and also facilitates the maternal-young relationship bond, which is important for neonate survival and its subsequent behavioral and physiological development (Hofer, 1994). Recognition also plays a pivotal role in the offspring's later socialization and attachment (Smith, Van-Toller, & Boyes, 1966). Thus, recognition is essential not only in maintaining the cost-effectiveness of maternal investment in offspring care but also in ensuring the survival and development of the offspring.

Early development of recognition is very important in species with precocial young, such as among ungulates, where maternal investment and energy expenditure is high and offspring are very mobile very quickly after birth (Morgan, Boundy, Arnold, & Lindsay, 1975). Among ungulates, individual recognition contributes to the rapid development of a maternal-infant relationship that is hard to break once established (Poindron, Lévy, & Keller, 2006). Formation of this relationship generally leads to resource allocation by the mother, promoting the likelihood of offspring survival. More specifically, ungulates are typically exclusive in allowing their own young near their udder (Smith et al., 1966). For example, Clutton-Brock, Albon, and Guinness (1989) explain that the cost of lactation is much greater than the cost of gestation in wild red deer, emphasizing the importance for the mothers to limit nursing to only their young. The ability of a mother to recognize her own young, therefore, is essential for her to establish this kind of selective nursing.

Similar to humans, auditory, visual, and especially olfactory cues are strongly associated with a maternal ungulate's ability to recognize her young. Olfactory signals are established very early on, for ewes (sheep) can identify their offspring's olfactory cues within minutes after parturition (Smith et al., 1966). The importance of olfactory cues is also emphasized in a study

by Ferreira et al. (2000), in which nursing behavior decreased among temporarily anosmic ewes right after parturition. Olfactory cues, however, are not always sufficient for recognition.

Among ungulates, recognition occurs on two levels: at short distances and at long distances (Searby & Jouventin, 2003; Terrazas, Ferreira, Lévy, Nowak, Serafin, Orgeur, Soto, & Poindron, 1999). Olfactory signals are only effective at short distances of 0.25 meters or less (Shillito & Alexander, 1975). Visual cues may not always be present and can be difficult to isolate, since sheep spend most of their time in large flocks. Therefore, at long distances, vocal recognition is likely to be of greatest importance, also serving as a fundamental factor in recognition at nearer distances. As a result, vocal discernment may be more important at farther distances for ungulates and can also be adequate for identification at closer distances.

Several studies on vocal recognition among ungulates reveal the significance of auditory signals on maternal behavior and suggest that vocalization is sufficient for ungulates and their young to recognize each other. The evolutionary benefits of this kind of recognition are clear. First, infant calls allow mothers to locate their young at a distance. This not only allows mothers to immediately respond to infant distress calls when separated, but also allows the mother to find the infant for nursing (Torriani, Vanonni, & McElligott, 2006). Illman et al. (2002) indicate that vocalization cues at a short range can potentially serve as a reinforcer with olfactory cues for offspring identification before allowing the infant to feed. Second, maternal calls allow infants of hider species to identify and locate their mothers at farther distances (Torriani et al., 2006). This facilitates an offspring's ability to seek its mother during danger and, more commonly, for nursing. There is little research on call recognition between siblings, but previous studies have suggested that sibling identification is also important, particularly for follower ungulates, such as

sheep (Nowak, 1990). The following section will provide a more in-depth analysis of the three types of vocal recognition relationships in sheep.

#### *Vocal Recognition of Lambs by Mothers*

The ability for a mother to recognize her offspring exclusively through auditory cues is extensively tested in sheep. Multiple studies conclude that vocalization is sufficient for recognition of lambs by their mothers (Ligout, Sebe, & Porter, 2003; Searby & Jouventin, 2003; Sèbe, Nowak, Poindron, & Aubin, 2007). There are several factors that demonstrate the importance and sufficiency of auditory signals. First, as a follower and highly social species that flocks in open fields, sheep stay in groups to avoid predation (Torriani et al., 2006). Consequently, it is important for ewes to recognize and locate their lambs at a distance during predator attacks. Sèbe, Nowak, Poindron, and Aubin (2007) speculate that ewes and lambs exhibit two types of calls: high pitched distressed calls and low pitched recognition calls. Ewes must recognize both calls to either locate offspring or for selective nursing to ensure proper maternal investment. Additionally, since they are a highly social species, follower ungulate mothers are constantly exposed to different recognition signals of their offspring. A study by Searby and Jouventin (2003) demonstrates through field playback tests that ewes show significantly greater response calls to their lambs than an alien sheep, suggesting that vocalization cues are sufficient for recognition. Finally, lambs will progressively spend more time in their peer groups apart from their mothers as early as three days of age (Morgan, Boundy, Arnold, & Lindsay, 1975). This makes auditory cues necessary for mothers to locate lambs for nursing, since distance recognition is more dependent on vocalization than olfaction and visual signals.

#### *Vocal Recognition of Mothers by Lambs*

Mother-young vocal recognition is fairly mutual in sheep, though it appears that mothers can discriminate auditory cues of their lamb and an unfamiliar lamb earlier than lambs can discriminate auditory cues of their mother from another ewe. More specifically, ewes can recognize their offspring's calls at 24 hours after birth while lambs do not recognize their mother's calls until 48 hours (Ligout et al., 2003). This may in part be due to the developmental ability of a lamb to recognize any sensory cue from the mother or to respond even if they recognize the call. Regardless, lambs hear their mother's voices immediately after birth, and probably before as well.

Following parturition, lambs are exposed to low-pitched rumbles from their mothers, often referred to as a "caregiver bleat" that is exclusive to a ewe's offspring (Dwyer et al., 1997). This early exposure helps with maternal-offspring bond formation and may also facilitate the lamb's ability to recognize its mother using only auditory cues. A two-choice test for mother discrimination at a distance of nine meters showed that lambs can identify their mothers without the help of olfactory cues by the age of three days (Nowak, 1990). This indicates that a combination of vocal and visual cues is sufficient for mother recognition. However, vocalization itself can be sufficient for recognition.

The selective maternal behavior of the ewe towards its offspring accentuates the need for offspring to identify their mothers to ensure nursing and survival (Nowak, 1990; Searby & Jouventin, 2003). After parturition, nursing ewes and lambs tend to aggregate into one group (Nowak, 1990). Such close contact can create overexposure to many olfactory and visual signals. Considering this problem, auditory cues may be more reliable in locating the correct mother for nursing. Moreover, considering lambs will spend more time apart from their mothers after three days of age, it is important for them to identify their mothers at a distance, particularly

when they want to nurse. According to Searby and Jouventin (2003), lambs between ages three days to two weeks will respond preferentially to their mothers' calls with behaviors such as return calls and body orients towards the direction of the call. This indicates that lambs are able to respond to the vocalizations of their mother. Thus, auditory cues are sufficient for mother recognition by lambs.

#### *Vocal Recognition between Siblings*

Some studies have shown that lambs appear capable of recognizing their siblings using only auditory cues. Recognition between siblings is more important in sheep. As a follower species, lambs not only follow their mother but also follow their mother and their twin or triplets (Nowak, 1990). This provides time for a sibling bond to establish. In addition, lambs will spend much of their time with their peer groups after three days of age (Nowak, 1990), which again provides them with more opportunity to form a bond with their siblings and be exposed to recognition cues. Finally, lamb siblings tend to spend more time with each other than with other age mates. This association is clearly demonstrated upon weaning, in which twin lambs spend an increased amount of time with each other than with other lambs for the first two days (Shillito-Walser & Williams, 1985). Accordingly, the amount of time lambs spend with each other coupled with their behavior during weaning illustrates the importance and ability of sibling mutual recognition.

Several studies on vocal recognition appear to confirm that auditory cues are sufficient for mutual sibling identification. One study by Shillito-Walser, Hague, and Yeomans (1982) verifies sibling preference through live preference tests, in which lambs were given the option to choose their sibling or a non-sibling in a T-maze. Results from this study indicate that Jacob lambs as young as three weeks old spent more time with their sibling than the alien lamb. More



importantly, the sibling lambs bleated less once they were near each other, while the alien lamb continued bleating, suggesting that the sibling lambs were bleating in response to each other's calls until they were reunited. This same result was illustrated in a different study, in which twin pairs bleated less than non-twin pairs when isolated from the flock and placed in a pen together (Ligout & Porter, 2002). In addition, another study by Ligout et al. (2003) used playback tests by to reveal that lambs will bleat more in response to their sibling's calls than to an unfamiliar lamb, again indicating that they recognize their sibling using only auditory cues. In summary, the results of these studies provide data that support the hypothesis that sibling lambs recognize each other based on their vocalizations.

Currently, there is very little research on behavioral sex differences in recognition ability, much less vocal recognition ability, of ungulates in the three types of recognition relationships previously described. Of the relationships, there are even fewer studies on sex differences on the mutual vocal recognition ability between siblings. Data on association patterns of young sheep show that female lambs spend more time with their mother and their sibling than do male lambs (Dwyer & Lawrence, 1999; Padmanabhan, Lee, & Coolen, 2008). This suggests that males are less motivated to associate with close relatives or that they do not discriminate between kin and non-kin as well as female lambs. Sex differences in early behavior are often caused by prenatal exposure to testosterone in male lambs.

Finally, there are no studies observing prenatal androgen effect on sheep recognition relationships. In my research, I asked whether the sex and prenatal androgen exposure of the lamb impacts the response to calls from related and unrelated sheep. If vocal recognition does vary, the maternal-young and sibling-sibling bond may also vary.

In this study, I used playback tests to assess three hypotheses. First, I hypothesized that lambs will not only be able to discriminate between mother and familiar non-maternal ewe calls, they will also be able to discriminate between sibling and familiar non-sibling calls. Second, I hypothesized that there is a sex difference in the ability to discriminate between kin and non-kin using only auditory cues, with females and controls showing a higher preference than males and t-treated lambs for kin calls over familiar non-kin calls. Finally, I hypothesized that female lambs exposed to prenatal testosterone will act more like control males in their response to playback tests.

## Method

### *Subjects*

Sixty-four female Suffolk sheep (28 from 2007, 36 from 2008) were obtained for this experiment after the approval of the University Committee on Use and Care of Animals (UCUCA) and the University of Michigan. The ewes were bred between October and December of 2007 and 2008 and gave birth to lambs between March and May for each year. The ewes were randomly split into two treatment groups during their pregnancy and were given either testosterone (T) or remained as control (C) groups. During their 30-90 day critical period of their 147 day-gestation, the mothers of the T-treated group were given intramuscular (i.m.) injections of 100mg of T in 2 mL cottonseed oil twice each week. The mothers of the control group received i.m. injections of 2 mL cottonseed oil twice each week.

A total of 27 C-females (CF), 16 C-males (CM), 19 T-females (TF), and 21 T-males (TM) were used for the mother/familiar ewe playback test (mother test) and 22 C-females, 16 C-males, 21 T-females, and 17 T-males were used for the sibling/familiar non-sib test (sibling test). There were 14 twins, 2 triplets, and 4 single lambs for the control group and 16 twins, 2 triplets,

and 3 single lambs for the T-group that underwent the sibling test. Single lambs in the sibling test had twins that were either not tested or included in this research study for various reasons. Over all, a total of 83 lambs were used in one or both playback tests.

In each year, the lambs were reared by their mothers following birth and weaned at 8 to 10 weeks. The lambs were kept in close contact with facility and research personnel during their infancy period to develop comfort towards humans. They were given a daily diet of hay, corn, and alfalfa pellets. Water was given to them *ad libitum*. All the sheep were housed at the University of Michigan Sheep Research Facility.

### *Materials*

*Vocalization collection.* At least two to three calls from the mom, the sibling(s), and unfamiliar ewes and lambs were collected using a portable digital recorder (Marantz Professional PMD660, D&M Holdings Inc., 2006) one to two weeks prior of testing. Call collections were then uploaded onto Raven Lite 1.0, where the best quality call for each mom and sibling was then extracted from the recording file and converted into an mp3 file. Next, audio clips for each ewe and lamb used in testing were designed in Audacity 1.2 and saved as mp3 files. Audio clips were two minutes and 20 seconds long, beginning with a 20 second period of silence. Following the silence was a call (call 1), which was either the sib or non-sib. The same call played again after 5 seconds of silence, before another 25 seconds of silence. The next call (call 2) was from the other animal, which also played twice. Both pairs of calls were played one more time in the same order and structure, for a total of two sets of calls from each source animal. A diagram for the audio clip design is shown in Figure 1.

*Experiment set-up.* The mother and sibling call recognition tests took place in a 17 ft by 16.5 ft indoor room. A researcher with a handheld digital video camera was positioned in the

middle of the wall opposite the entrance. One speaker was placed to the left and to the right of the researcher, both connecting to an Apple Ipod containing files of audio recordings for the playback tests. The researcher and the speakers were blocked from the view of the lamb using panels covered with drapes. A diagram of this room is shown in Figure 2.

### *Procedure*

Testing began by releasing an individual lamb into the experiment room as the researcher starts the audio recording. Recorded vocalizations of the sib and a randomly selected, familiar non-sib panned through the left and right speakers, respectively. Assignment of speaker side and order of call for sibs and non-sibs were randomly chosen for each test animal.

Behavior of the test animal was recorded with the video camera for the duration of the audio clip. Measures included responses such duration spent freezing to the call; number of orients toward each speaker; small, medium, and large approaches made towards each speaker; investigate and jumping onto the speaker; bleat frequency; and duration spent in the near, medium, and far areas from the speaker. The behavioral measures listed are described in Appendix A. All behaviors were recorded in relation to which speaker side the sib call was coming from.

The mother/familiar ewe tests were structured the same way as the sib test, using mom and familiar ewe recordings for the speakers.

### *Data Collection*

Lambs were tested at approximately 2 to 4 weeks of age between mid-April and late-June, depending on date of birth, for both the mother and sib test. Video data of the behavior gathered from the mother test and sib test were uploaded onto a Mac computer. Files were played in iMovie and behaviors were recorded onto an excel spreadsheet. To avoid biased

coding, coders were blinded from information indicating which speaker side the kin call was coming from and treatment and sex of lambs being tested.

General linear models and ANOVA models were used to analyze variance and significance levels of sex and treatment effects. The statistical analyses were performed with Systat Version 10 Copyright SPSS Inc., 2000.

## Results

For both the mother and sibling playback tests, lambs demonstrate an ability to discriminate behaviorally discriminate the kin and non-kin calls. Their response behaviors (freeze, orient, approach, investigate, jump, bleat frequency, and area duration, or time spent near the speaker of their mother, sibling, or familiar sheep) were more clearly differentiated between kin and non-kin, treatment groups, and sex for the ewe test than the sibling test. The following is a more specific discussion of the results for each test.

### *Mother Playback Test*

Analysis of playback tests showed that lambs exhibit significantly different response behaviors towards the mother speaker than the familiar ewe (not-mother) speaker.

*Freeze.* All test lambs spent a significantly longer time freezing in response to mother calls ( $M = 1.537$ ,  $SEM = 0.391$ ) compared to not-mother calls ( $M = 0.750$ ,  $SEM = 0.254$ ), ( $F[1, 158] = 9.627$ ,  $p = 0.002$ ), reflecting a differentiated response to the type of call. This difference was observed in each sex by treatment group as well, with TM freezing longest, followed by CF, TF, and CM,  $F(1,158) = 6.911$ ,  $p = 0.009$  (see Figure 3). There were no significant differences between sexes. However, when analyzing responses for each speaker side, there was a significant sex by treatment effect for familiar ewe freezing ( $F[1,79] = 6.096$ ,  $p = 0.016$ ), with TM freezing the most and CM freezing the least (see Figure 4). Additionally, T-treated lambs

froze longer than controls in response to familiar ewe calls,  $F(1,79) = 0.067$ ,  $p = 0.067$ . In general, T-treated lambs froze significantly longer to calls, regardless of source, than control lambs ( $M_T = 0.881$ ,  $SEM = 0.278$ ;  $M_C = 1.406$ ,  $SEM = 0.367$ ),  $F(1,158) = 4.305$ ,  $p = 0.040$ .

*Orient.* All test lambs also oriented significantly more to mother calls familiar ewe ( $M_M = 2.5$ ,  $SEM = 0.324$ ;  $M_{FE} = 2.22$ ,  $SEM = 0.300$ ), ( $F[1,158] = 4.827$ ,  $p = 0.029$ ), again showing that their responses were differentiated between the type of call. Sex differences were not significant, though there was a treatment trend ( $F[1,79] = 2.826$ ,  $p = 0.097$ ) for orients toward the mother, with T-treated ( $M = 2.782$ ,  $SEM = 0.324$ ) orienting more than controls ( $M = 2.218$ ,  $SEM = 0.324$ ). Finally, similar to the results for freezes, T-treated lambs ( $M = 2.70$ ,  $SEM = 0.307$ ) oriented more frequently to a significant degree ( $F[1,158] = 4.405$ ,  $p = 0.037$ ) to any ewe's call compared to control lambs ( $M = 2.016$ ,  $SEM = 0.318$ ).

*Approach.* All test lambs made small approaches toward the mother speaker significantly more often than to the familiar ewe speaker,  $F(1,158) = 3.909$ ,  $p = 0.050$  (see Figure 5). Lambs also made more medium and large approaches toward the mother speaker than familiar ewe, though not significantly (see Figure 5). Moreover, there was a significant treatment effect ( $F[1,79] = 6.485$ ,  $p = 0.013$ ) for small approaches made towards the mother speaker, with T-treated lambs exhibiting more large approaches than controls (see Figure 6). Sex effects were insignificant across all approach types.

*Investigate.* Similar to freeze, orient, and approach, all test animals investigated the mother speaker ( $M = 0.663$ ,  $SEM = 0.263$ ) significantly more than the familiar ewe speaker ( $M = 0.282$ ,  $SEM = 0.117$ ),  $F(1,158) = 5.225$ ,  $p = 0.024$ . Furthermore, there was a sex effect trend for investigate counts ( $F[1,158] = 3.123$ ,  $p = 0.079$ ), with females investigating the mother speaker more often than the familiar ewe speaker ( $M_M = 0.939$ ,  $SEM = 0.369$ ;  $M_{FE} = 0.264$ ,  $SEM$

= 0.109) compared to males ( $M_M = 0.387$ ,  $SEM = 0.158$ ;  $M_{FE} = 0.3005$ ,  $SEM = 0.125$ ). There was also a trend for females to investigate the mother speaker more often than the males  $F(1,79) = 3.266$ ,  $p = 0.075$ . When breaking down the results by sex by treatment, there was a significant effect for familiar ewe investigations ( $F[1,79] = 4.517$ ,  $p = 0.037$ ), with TM investigating the most (see Figure 7 ).

*Jump.* There were no significant differences between treatment groups, sex, and sex by treatment groups. This is partially due to the rarity of such a behavior during the test.

*Bleat Frequency.* Test lambs did not display differentiated bleating frequency in response to mother and familiar ewe calls. However, there was a near significant treatment difference for familiar ewe bleat frequency of ( $F[1,78] = 3.354$ ,  $p = 0.071$ ), with controls bleating more than T-treated at an average of 0.215 bleats/s ( $SEM = 0.014$ ) compared to 0.197 bleats/s ( $SEM = 0.017$ ) after they hear the call. There was also a significant treatment effect ( $F[1,158] = 5.636$ ,  $p = 0.019$ ), with controls ( $M = 0.216$ ,  $SEM = 0.015$ ) bleating more frequently than T-treated ( $M = 0.186$ ,  $SEM = 0.015$ ) after the calls.

*Area Duration.* The time spent near or far away from the mother and familiar ewe were significant ( $F[1, 158] = 5.905$ ,  $p = 0.016$ ;  $F[1,158] = 9.144$ ,  $p = 0.003$ , respectively). Test lambs spent more time near their mother than the familiar ewe and spent more time far from the familiar ewe than their mother (see Figure 8). When combining duration spent in near and middle areas, which are the areas closest to the speaker, there was a very significant difference in time spent in near + middle mother and near + middle familiar ewe speaker areas  $F(1,158) = 9.306$ ,  $p = 0.003$ . More specifically, all animals spent more time near the mother ( $M = 92.906$ ,  $SEM = 11.202$ ) than familiar ewe ( $M = 81.211$ ,  $SEM = 9.966$ ).

There was also a significant treatment effect for duration spent in the middle area ( $F[1,158] = 6.499, p = 0.012$ ), with controls spending more time near the mother than the familiar ewe and T-treated doing the opposite (see Figure 9). Also, T-treated spent more time near the mother than controls,  $F(1, 79) = 5.184, p = 0.026$  (see Figure 10). Finally, T-treated spent more time in the middle familiar ewe area than controls,  $F[1,79] = 3.972, p = 0.050$ . On the other hand, controls spent that missing time far from the familiar ewe, which was longer than the time T-treated spent there,  $F(1,79) = 2.830, p = 0.096$  (see Figure 11).

There was a significant sex difference in duration spent far from the mother compared to the familiar ewe,  $F(1,158) = 4.233, p = 0.041$ . The difference spent between the two areas was greater in males than females, with both spending more time far from the familiar ewe than the mother. When combining near and mid areas, there was a significant sex effect on time spent in each area for mother and familiar ewe,  $F(1,158) = 4.308, p = 0.040$ . Both sex groups spent more time in the near + middle areas for mother over the familiar ewe, with a greater difference in time spent between the two areas in males than females. Males also spent a significantly longer time than females far from the familiar ewe  $F(1,79) = 4.900, p = 0.030$ . Females spent more of their time in the middle areas for both speakers (see Figure 12).

### *Sibling Playback Test*

Analysis of the playback tests showed that lambs were not as differentiated in their response behaviors toward sib and non-sib calls compared to the mother playback tests. However, significant responses toward the sib and non-sib speakers showed that controls made more behavioral responses after sibling calls than T-treated, and females more than males.

*Freeze.* Freeze duration between sib and non-sib calls for all test lambs were not significantly different, though lambs did spend more time freezing toward the sib speaker ( $M =$



0.679,  $SEM = 0.297$ ) than non-sib ( $M = 0.609$ ,  $SEM = 0.295$ ). Treatment effect on freeze duration was significant for sib calls ( $F[1,72] = 7.830$ ,  $p = 0.007$ ), with controls freezing longer than T-treated ( $M_C = 1.128$ ,  $SEM = 0.431$ ;  $M_T = 0.231$ ,  $SEM = 0.163$ ). Additionally, controls ( $M = 0.975$ ,  $SEM = 0.386$ ) froze significantly longer than T-treated lambs ( $M = 0.314$ ,  $SEM = 0.206$ ) in response to lamb calls in general, regardless of kinship to the animal,  $F(1, 144) = 9.129$ ,  $p=0.003$ . There were no sex differences for freeze duration for the sib test.

*Orient.* Similar to freeze response, orient was not significantly different between the sib and non-sib calls, though all test lambs did orient more to the sib than non-sib speaker. There was a trend in treatment effect for orients toward the sib speaker ( $F[1,72] = 3.140$ ,  $p = 0.081$ ), with controls orienting more frequently than T-treated ( $M_C = 2.208$ ,  $SEM = 0.236$ ;  $M_T = 1.740$ ,  $SEM = 0.289$ ). Controls also oriented more than T-treated in response to non-sib calls, though not significantly more. Over all, orient counts were higher for controls ( $M = 0.975$ ,  $SEM = 0.386$ ) than T-treated ( $M=0.314$ ,  $SEM = 0.206$ ) for both speakers  $F(1,144) = 3.052$ ,  $p = 0.083$ . Again, there were no significant sex effects for this behavior.

*Approach.* There were no significant differences in number of approaches made toward the sib versus non-sib speakers for test lambs. However, there was a significant sex effect on large approaches made toward the sib and non-sib speakers  $F(1,144) = 4.595$ ,  $p = 0.034$ . Females made more large approaches toward the sib speaker than non-sib speaker ( $M_S = 0.161$ ,  $M_{NS} = 0.208$ ), while males made more large approaches toward the non-sib speaker than sib speaker ( $M_{NS} = 0.155$ ,  $M_S = 0.429$ ). On the other hand, when comparing number of approaches made toward each speaker, females made significantly more large approaches toward the non-sib speaker than males,  $F(1,72) = 5.589$ ,  $p = 0.021$  (see Figure 13). Females also made significantly more medium approaches toward the non-sib speaker than males  $F(1,72) = 7.222$ ,  $p = 0.009$  (see

Figure 13). For approaches made toward speakers, regardless of the animal, females made more medium approaches than males ( $M_F = 0.640$ ,  $SEM = 0.159$ ;  $M_M = 0.380$ ,  $SEM = 0.135$ ),  $F(1,144) = 5.594$ ,  $p = 0.019$ . There were also trends and significance in results on treatment effect. For instance, controls made large approaches toward the sib speaker more frequently than T-treated  $F(1, 72) = 3.141$ ,  $p = 0.081$  (see Figure 14). Furthermore, controls made more medium approaches toward sib than non-sib ( $F[1,144] = 3.240$ ,  $p = 0.074$ ) and made more large approaches toward any lamb speaker than T-treated  $F(1,144) = 5.824$ ,  $p = 0.017$ .

*Investigate.* There were no significant differences across all lambs, treatment groups, sex groups, and sex by treatment groups. This is partially due to the rarity of such a behavior during the test.

*Jump.* Jump count differences were not significant over all, though differences did appear between treatment groups, with a trend for controls ( $M = 0.253$ ,  $SEM = 0.137$ ) to exhibit the behavior more than T-treated ( $M = 0.104$ ,  $SEM = 0.080$ ),  $F(1,144) = 3.037$ ,  $p = 0.084$ . In particular, control lambs made more jumps than T-treated lambs for the sib and the non-sib speaker, though the difference only approached significance for the sib speaker ( $M_C = 0.284$ ,  $SEM = 0.159$ ;  $M_T = 0.077$ ,  $SEM = 0.063$ ),  $F(1,72) = 3.109$ ,  $p = 0.082$ .

*Bleat Frequency.* Bleat frequency was not significantly different over all, though there was a trend for treatment by sex effect ( $F[1,144] = 3.018$ ,  $p = 0.084$ ) for total bleating frequency toward both speakers, with CM bleating the most, followed by CF, TF, and TM (see Figure 15). All controls bleated more than T-treated, though again, it is not significant.

*Area Duration.* The combined results of all test lambs did not show significant area duration differences between speaker sides. However, when accounting for treatment differences, there was a trend in treatment effect on speaker side preference ( $F[1,144] = 3.579$ ,  $p$

= 0.061), in which controls spent more time in the area nearest to the sib speaker than the non-sib speaker, while T-treated did exactly the opposite (see Figure 16). There was also a significant treatment effect for duration in the middle non-sib area, with controls spending more time there than T-treated,  $F(1,72) = 4.352$ ,  $p = 0.041$  (see Figure 17). Finally, there were no significant sex effects on area duration.

## Discussion

Playback tests in this study confirmed the hypothesis that auditory cues are sufficient for kin recognition by lambs. For both tests, lambs were able to discriminate to a certain degree between their mothers and sib from familiar not-mothers and familiar non-sibs. Furthermore, being able to discriminate between kin and familiar sheep, as opposed to unfamiliar sheep, demonstrated that lambs had a high level of discrimination. In addition, at 2 to 4 weeks, lambs are more likely to recognize and respond to calls of their mother than to calls of their sibling.

Treatment effects did not completely fit the hypothesis for the mother test. My hypothesis was that controls would respond to mother calls more than T-treated, while T-treated females would act more like control males. However, T-treated responded more than controls for some measures and there were no sex differences. Treatment effects were as predicted for the sib test, though the fact that the behavioral responses were the opposite from the mom test was unexpected. Finally, the sex effects for both tests were not as significantly different as I predicted. Over all, sex effects were unclear and less defined compared to treatment effects.

The following is a more specific interpretation of the results for each test.

### *Mother Playback Test*

All lambs, regardless of sex or treatment, exhibited responsive behaviors that occurred more frequently (orient, approach, and investigate) or occurred for a longer duration of time

(freeze, area duration) toward the mother call. This showed not only an ability to discriminate between the mother and familiar ewe using auditory cues, but also shows a preference toward the mother. Thus, when provided with only auditory signals, lambs would freeze longer, orient, approach, and then investigate the speaker of their mother over an familiar ewe. They ultimately chose to stay closer to the mother for the duration of the test.

Treatment effects were very clear for the mother playback test, with T-treated lambs responding more to sib and non-sib calls than controls (freeze, orient, approach, investigate, area duration). Such results confirms previous research, which have shown that prenatal T increases masculine behaviors in humans and mammals (Berenbaum & Resnick, 1997; Dittmann et al., 1990; Hines, 2008; Manson, 2008; Mathews et al., 2009; Meaney & McEwen, 1986; Pasterski et al., 2007; Roberts, Padmanabhan, & Lee, 2008). These behaviors include exhibiting more curious and exploratory behaviors in isolated situations (Øverli, Sørensen, Pulman, Pottinger, Korzan, Summers, & Nilsson). This is specifically demonstrated through behaviors the T-treated groups made toward the familiar ewe speaker. Additionally, considering that sheep are social animals and do not prefer to be isolated from their flock, it is likely that control animals were more fearful. For example, previous studies had suggested that prenatal T decreases stress response in mildly stressful situations (Padmanabhan, Lee, & Coolen, 2008). Therefore, they would be less likely to respond to their mother's calls. Furthermore, bleating frequencies between T-treated and controls can be interpreted in two ways. First, considering T-treated animals froze for a longer duration than controls, it is likely that this contributed to their reduced bleating frequency, since lambs were silent when freezing. Second, previous research has shown that lambs stopped bleating in isolated situations when they find their siblings, showing that they were no longer stressed (Ligout & Porter, 2002, Shillito-Walser, Hague, & Yeomans, 1982).

This could have been possible for T-animals, whose behavior indicated that they not only indentified their mothers but responded to their mother's calls. Thus, though both groups showed behaviors indicating their ability to discriminate between mother and familiar ewe calls, T-treated animals were more likely to approach the call sources and explore the testing area, while controls were more likely to exercise caution in responding to the calls.

Overall, sex effects were not very clear for this test. This may be due to the fact that both tests were performed before lambs reached puberty, when sex specific behaviors become more prominent. On the other hand, they do confirm the idea of T-treated animals being more aggressive and less cautious with their curiosity. While females investigated the call sources more than males, males were more likely to investigate the familiar ewe speaker, possibly due to curiosity and courage. Furthermore, males demonstrated that, even though they would respond to familiar ewe calls, they preferred their mother's calls by spending more time in the near + middle mother area than near + middle familiar ewe area. They also spent more time away from the familiar ewe, despite investigating that area more often. Females, on the other hand, did not spend a significant time in either near + middle areas. They instead spent most of their time in the middle areas, possibly due to fear of approaching the speaker area.

#### *Sibling Playback Test*

Response behaviors in the sib test were not as clearly differentiated between kin and non-kin, treatment groups, and sex. One possibility for this result is that both tests were performed prior to weaning of the lambs from their mothers. Thus, their ability to discriminate between sib and non-sib calls may not have been as important compared to their ability to discriminate between mother and familiar ewe calls. Studies have also shown that following weaning, the absence of their mothers make lambs spend more time with their siblings (Shillito-Walser &

Williams, 1985). Therefore, we should re-examine sibling discrimination in recently weaned lambs.

The significant effects that were present during the playback test showed the opposite behaviors compared to the mother test. For instance, controls were more likely to respond to the speakers than T-treated. T-treated lambs generally showed fewer responses toward any speaker, regardless of kin and non-kin calls. This indicates a dampened response to calls in general, as well as a decreased degree of discrimination between the sib and non-sib calls. These results are consistent with past studies on prenatal T effect on association patterns (Dwyer & Lawrence, 1999, Padmanabhan, Lee, Coolen, 2008). One possibility is that, if sibling call recognition is unnecessary at 2 to 4 weeks, testosterone may actually increase the likelihood for lambs to respond to their mother's calls. In other words, at 2-4 weeks, testosterone increases lamb reactivity to their mother's calls and decreases reactivity to their sibling's calls. Finally, sex effects were also the opposite from the mother playback test, with females responding to calls more than males. This follows the same pattern as the treatment effect. In the mother test, T-treated and males showed more masculinized behaviors. In the sib test, T-treated animals and males showed less masculinized behaviors. However, for the most part, sex effects were not significant, which reinforces the idea that sexually differentiated behavior does not arise until after weaning.

The overall results for this research confirm previous research that lambs are able to recognize their mother using only auditory cues (Ligout et al., 2003; Searby & Jouventin, 2003). The results also show that lambs are able to recognize their siblings using only auditory cues, though not as well as they do with their mothers at 2-4 weeks. Both tests prove that lambs have vocal recognition ability regardless of sex and treatment. Treatment and sex effects were flipped

between the mother and sibling playback test, though such a difference can be explained by the age of the lambs and the idea that weaning may strengthen the sibling recognition once the mom is removed. Contrary to my initial hypothesis, T-treated females did not act more like control males in both tests, partially because there were few significant sex effects for the behaviors and also because all T-treated animals acted more like control females in the sibling test.

Besides the age of the lambs in which this test was performed, one issue of the test was that behavioral responses may have occurred in response to fear of being in an isolated situation as well as in response to recognition of kin vocalizations. For instance, results of the mother test are similar to studies of stress reactivity of sheep in mildly stressful situations, including isolation (Padmanabhan, Lee, Coolen, 2008). Thus, it may be more effective to analyze the recognition ability of lambs using only auditory cues in settings where they are not forced into an isolated situation. One previous study Searby and Jouventin (2003) observed response to recorded playbacks while lambs were alone in the pasture. While this is more difficult to perform and cannot ensure the isolation of lambs, behavioral responses to just mother and sibling calls can be more accurate.

The next steps for this study are to analyze the data that has already been collected in my lab for sibling tests performed post-weaning in order to see if there is a greater response to sibling calls after the lamb is weaned from its mother. Analyzing data at a later age may also reveal more clearly differentiated sex effects. We will also compare the results of the playback tests with the results of live sibling preference tests, in which lambs are placed in a room where they can choose between their sib and a familiar non-sib to confirm that siblings are able to identify each other when all recognition cues are present and also observe if treatment and sex effects are the same compared to the vocal recognition tests when the isolation factor is removed.

Finally, to ascertain a more accurate assessment of the effect of T on kin vocal recognition, we should compare the behavioral responses in call playback tests of control and T-treated animals to animals of other treatment groups, such as dihydrotestosterone (DHT), estrogen (E), and flutamide. Currently, research shows that prenatal exposure to DHT causes virilization of physical traits and demasculinization of behavior, while flutamide causes the reverse (Thornton, Irvin, & Goy, 1989; West, Foster, Evans, Robinson, & Padmanabhan, 2001; Goto, Koizumi, Takaori, Fujii, Furuyama, Saika, Saito, & Suzuki, 2004). We currently have playback test data on animals exposed to prenatal DHT and flutamide, which we will analyze to compare with our current data on T and controls. We do not have data on prenatal E, but should include it in our future comparisons, as prenatal E can cause defeminization of behavioral traits (vom Saal, Grant, McMullen, & Laves, 1983). Comparing across more treatment groups will offer a better perspective on the effects of prenatal testosterone recognition based on auditory cues. Thus, by including more treatment groups and analyzing data from tests performed at later age time points, we may be able to observe greater treatment and sex effects on kin and non-kin call recognition ability of lambs.



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## Appendix A: Vocalization Test Ethogram.

<b>Term</b>	<b>Definition</b>
Silence Interval	The 20 second period in which no calls are played. Silence intervals follow the call interval every 2 calls that are played. Behavior during this interval is coded based on the speaker side of the last call.
Call Interval	The 5 second period in which a call is played.
Animal 1	This animal's calls are played first. These calls are labeled Call 1.
Animal 2	This animal's calls are played second. These calls are labeled Call 2.
Freeze	An immediate response behavior following the call. Lamb stops moving and/or stiffens when call is played. Ears are up in response to sound and lamb is silent.
Orient	Lamb physically orients head to speaker playing the call.
Small Approach	Approach of only a few inches towards speaker playing the call. Lamb remains in the same area during approach.
Medium Approach	Approach of a few feet towards the speaker playing the call. Lamb still remains in the same area during the approach.
Large Approach	Approach of a few feet towards speaker playing the call. With this approach, the lamb will go from one area to the area closest to the speaker (LF or RF).
Bleats	Vocalization produced by lamb.
Bleat Frequency	Bleats/s that the lamb makes for the post-call and silence periods of each speaker.
Speaker Behavior	Codes for two types of behavior the lamb <i>may</i> do when in the zone closest to the speaker that played the call (LF or RF).
Investigate Speaker	Lamb physically touches speaker with nose, sniffs speaker, or paws speaker.
Jump Speaker	Lamb attempts to enter the speaker zone by placing front legs on the board in front of the speaker or by attempting to jump over board.
Near Area	The room is portioned into three areas. Near area is the area closest to the speaker (kin or non-kin).
Middle Area	Area in between the closest and farthest area. Lamb is approximately half a room's length from the speaker (kin or non-kin).
Far Area	Area farthest from the speaker (kin or non-kin).

## Author Note

Belinda Shih, Department of Psychology, University of Michigan, Ann Arbor.

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Correspondence concerning this article should be sent to Dr. Theresa Lee, Department of Psychology, University of Michigan, 1004 East Hall, Ann Arbor, MI, 48109-1109.



## Figure Captions

*Figure 1.* Playback test audio clip design.

*Figure 2.* Layout of the testing room. Room is divided into near, middle, and far areas to show how area is divided if the kin call is coming from the left speaker. Left speaker is labeled accordingly.

*Figure 3.* Mother test: Total freeze duration by sex by treatment. There was a significant sex by treatment effect for freeze response to all ewe calls ( $F[1,158] = 6.911, p = 0.009$ ), with T-treated males (TM) freezing the longest, followed by control females (CF), T-treated females (TF), and control males (CM).

*Figure 4.* Mother test: Freeze duration by sex by treatment. There was a significant sex by treatment effect for familiar, non-maternal ewe freezing ( $F[1,79] = 6.096, p = 0.016$ ), with TM freezing the most and CM freezing the least.

*Figure 5.* Mother test: Total approaches made for mother and familiar ewe. All test lambs made small approaches toward the mother speaker significantly more often than to the familiar, non-maternal ewe speaker,  $F(1,158) = 3.909, p = 0.050$ . Lambs also made more medium and large approaches toward the mother speaker than familiar, non-maternal ewe, though not significantly.

*Figure 6.* Mother test: Approach mother counts by treatment. There was a significant treatment effect ( $F[1,79] = 6.485, p=0.013$ ) for small approaches made towards the mother speaker, with T-treated lambs exhibiting more large approaches than controls.

*Figure 7.* Mother test: Investigate counts by sex by treatment. There was a significant effect for familiar, non-maternal ewe investigations ( $F[1,79] = 4.517, p = 0.037$ ), with TM investigating the most.

*Figure 8.* Mother test: Total mother and familiar, non-maternal ewe area durations. Test lambs spent more time near their mother than the familiar, non-maternal ewe ( $F[1, 158] = 5.905, p = 0.016$ ) and spent more time far from the familiar, non-maternal ewe ( $F[1,158] = 9.144, p = 0.003$ ) than their mother.

*Figure 9.* Mother test: Total mother and familiar, non-maternal ewe middle area duration by treatment. There was a significant treatment effect for duration spent in the middle area ( $F[1,158] = 6.499, p = 0.012$ ), with controls spending more time near the mother than the familiar, non-maternal ewe and T-treated doing the opposite.

*Figure 10.* Mother test: Mother and familiar, non-maternal ewe area duration by treatment. T-treated spent more time near the mother than controls,  $F(1, 79) = 5.184, p = 0.026$ .

*Figure 11.* Mother test: Familiar, non-maternal ewe area duration by treatment. T-treated spent more time in the middle familiar, non-maternal ewe area than controls,  $F[1,79] = 3.972, p = 0.050$ . Controls spent that missing time far from the familiar, non-maternal ewe, which was longer than the time T-treated spent there,  $F(1,79) = 2.830, p = 0.096$ .

*Figure 12.* Mother test: Familiar, non-maternal ewe area duration by sex. Males spent a significantly longer time than females far from the familiar, non-maternal ewe  $F(1,79) = 4.900, p = 0.030$ . Females spent more of their time in the middle areas for both speakers.

*Figure 13.* Sib test: Approach non-sib counts by sex. Females made significantly more large approaches toward the non-sib speaker than males,  $F(1,72) = 5.589, p = 0.021$ . Females also made significantly more medium approaches toward the non-sib speaker than males  $F(1,72) = 7.222, p = 0.009$ .

*Figure 14.* Sib test: Approach sib speaker counts by treatment. Controls made large approaches toward the sib speaker more frequently than T-treated  $F(1, 72) = 3.141, p = 0.081$ .

*Figure 15.* Sib test: Total bleat frequency by treatment by sex. There was a trend for treatment by sex effect ( $F[1,144] = 3.018, p = 0.084$ ) for total bleating frequency toward both speakers, with CM bleating the most, followed by CF, TF, and TM.

*Figure 16.* Sib test: Total sib and non-sib near area duration by treatment. there was a trend in treatment effect on speaker side preference ( $F[1,144] = 3.579, p = 0.061$ ), in which controls spent more time in the area nearest to the sib speaker than the non-sib speaker, while T-treated did exactly the opposite.

*Figure 17.* Sib test: Non-sib area duration by treatment. There was a significant treatment effect for duration in the middle non-sib area, with controls spending more time there than T-treated,  $F(1,72) = 4.352, p = 0.041$ .

Figure 1.

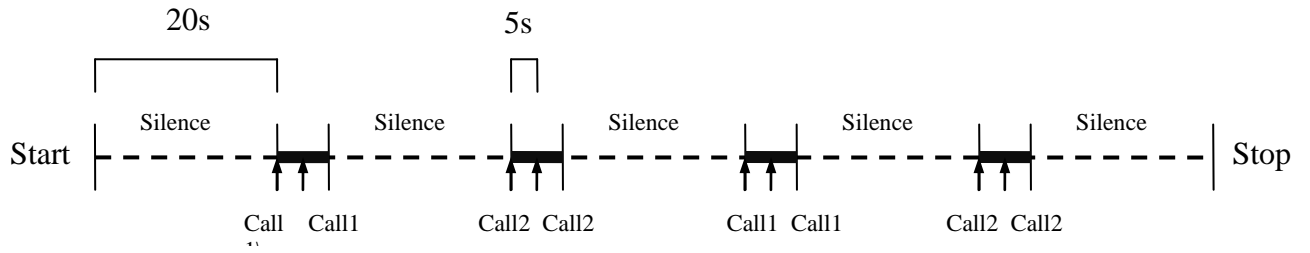


Figure 2.

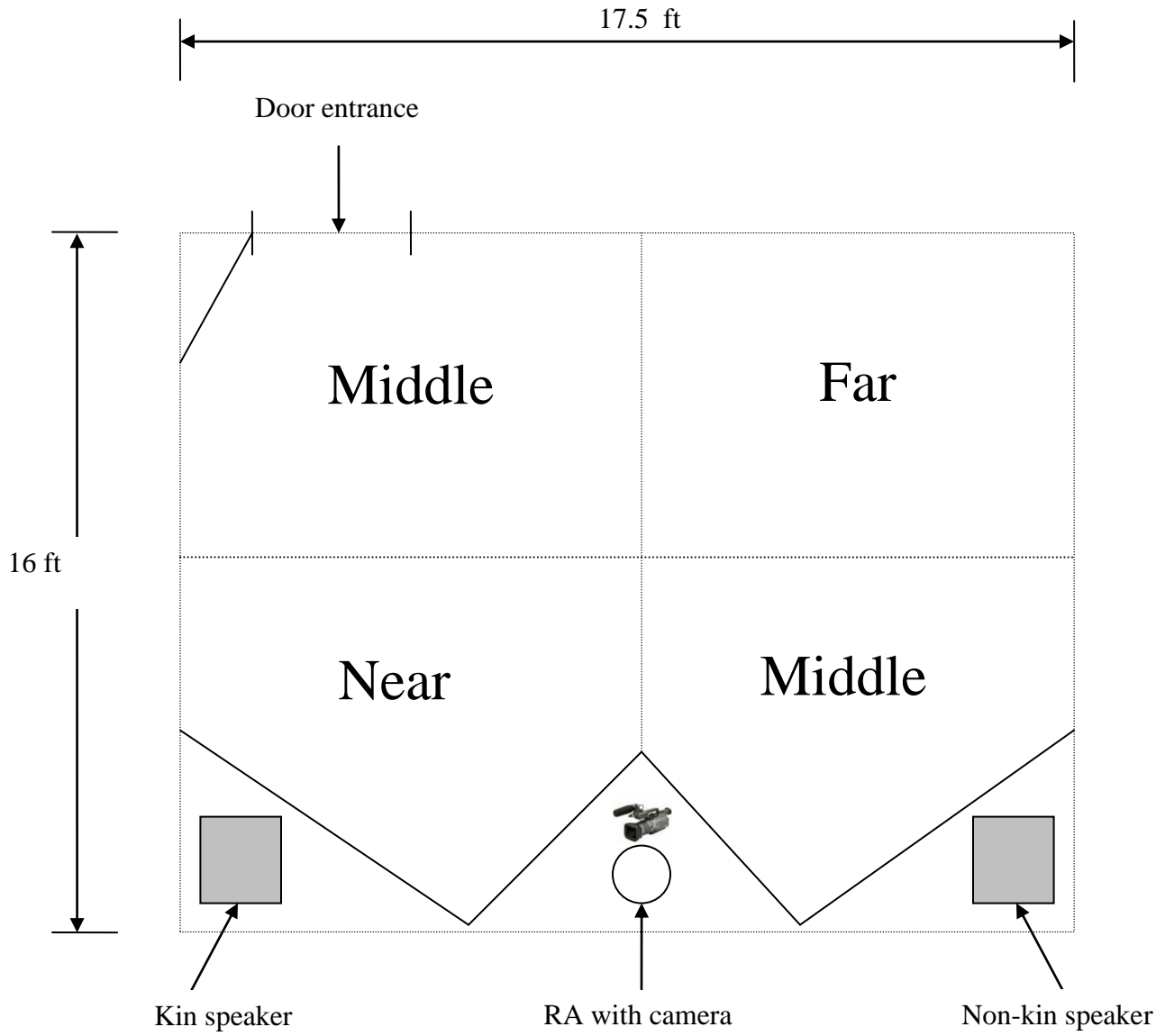


Figure 3.

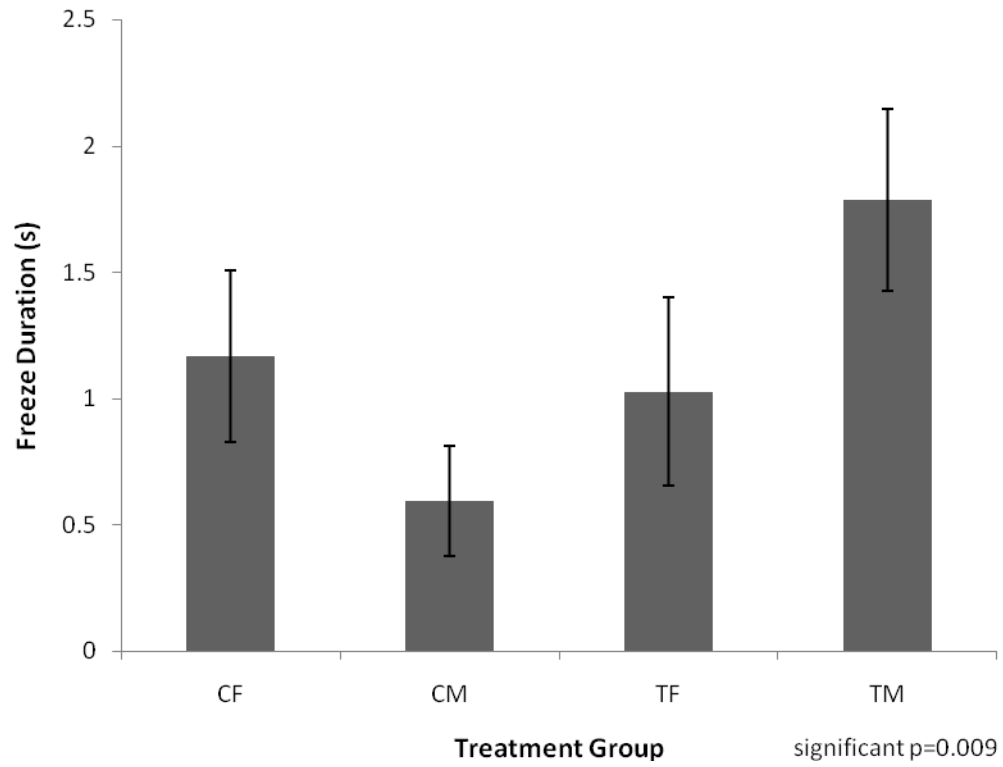


Figure 4.

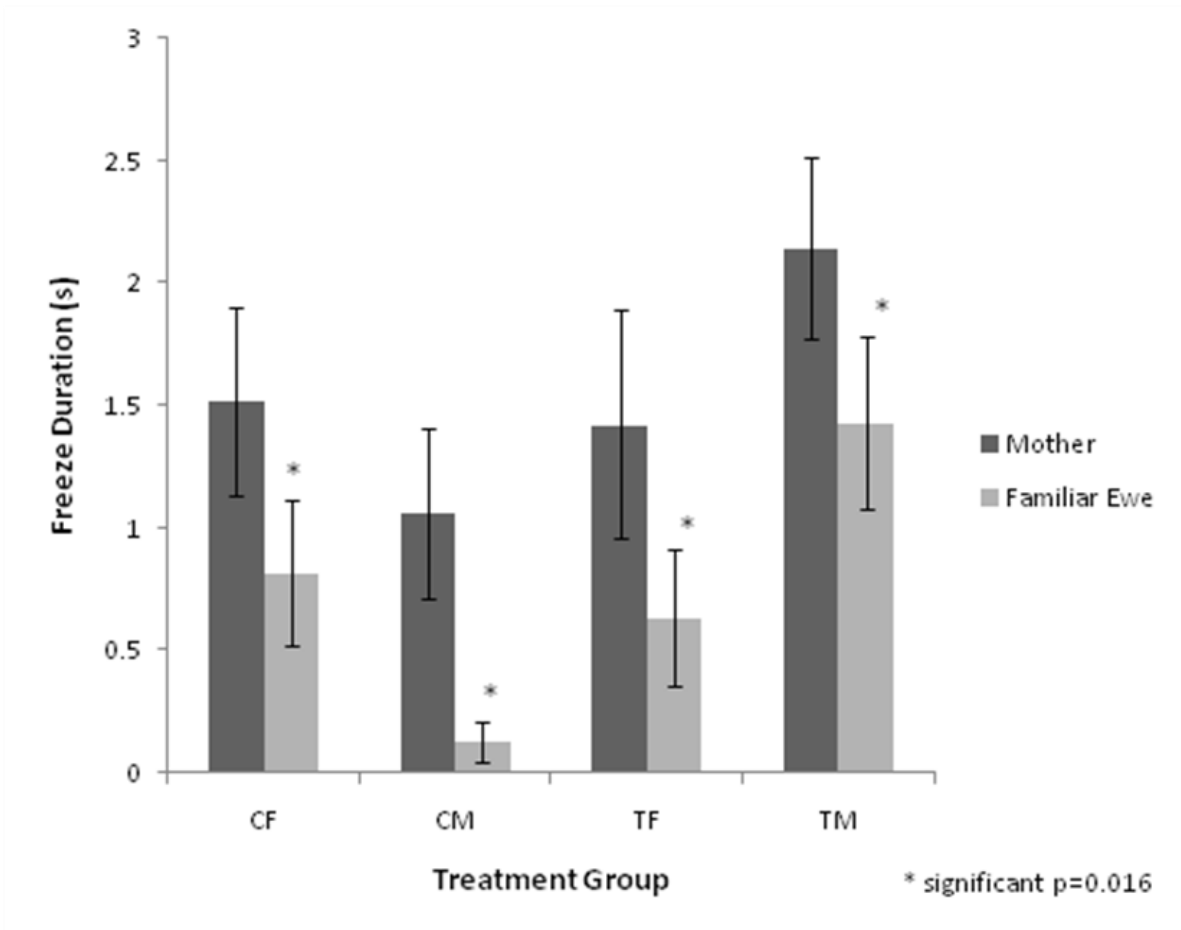


Figure 5.

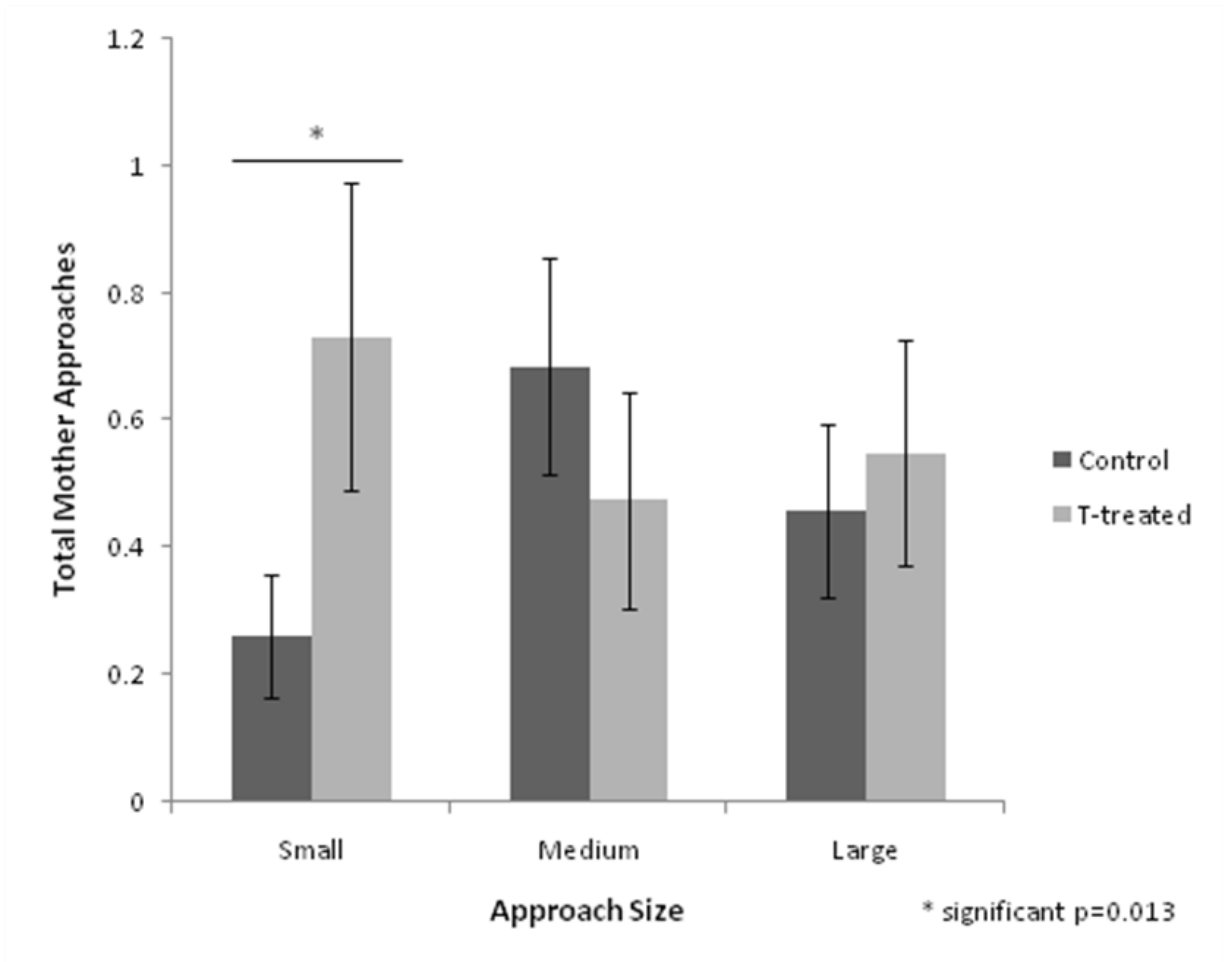




Figure 6.

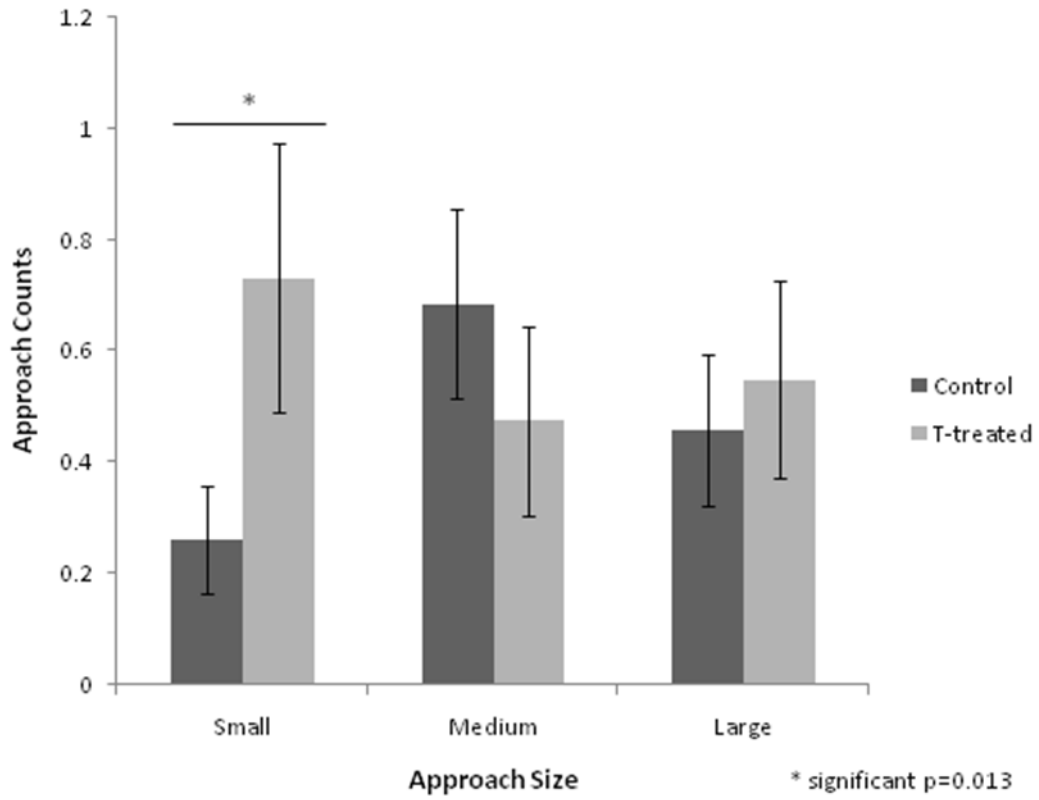


Figure 7.

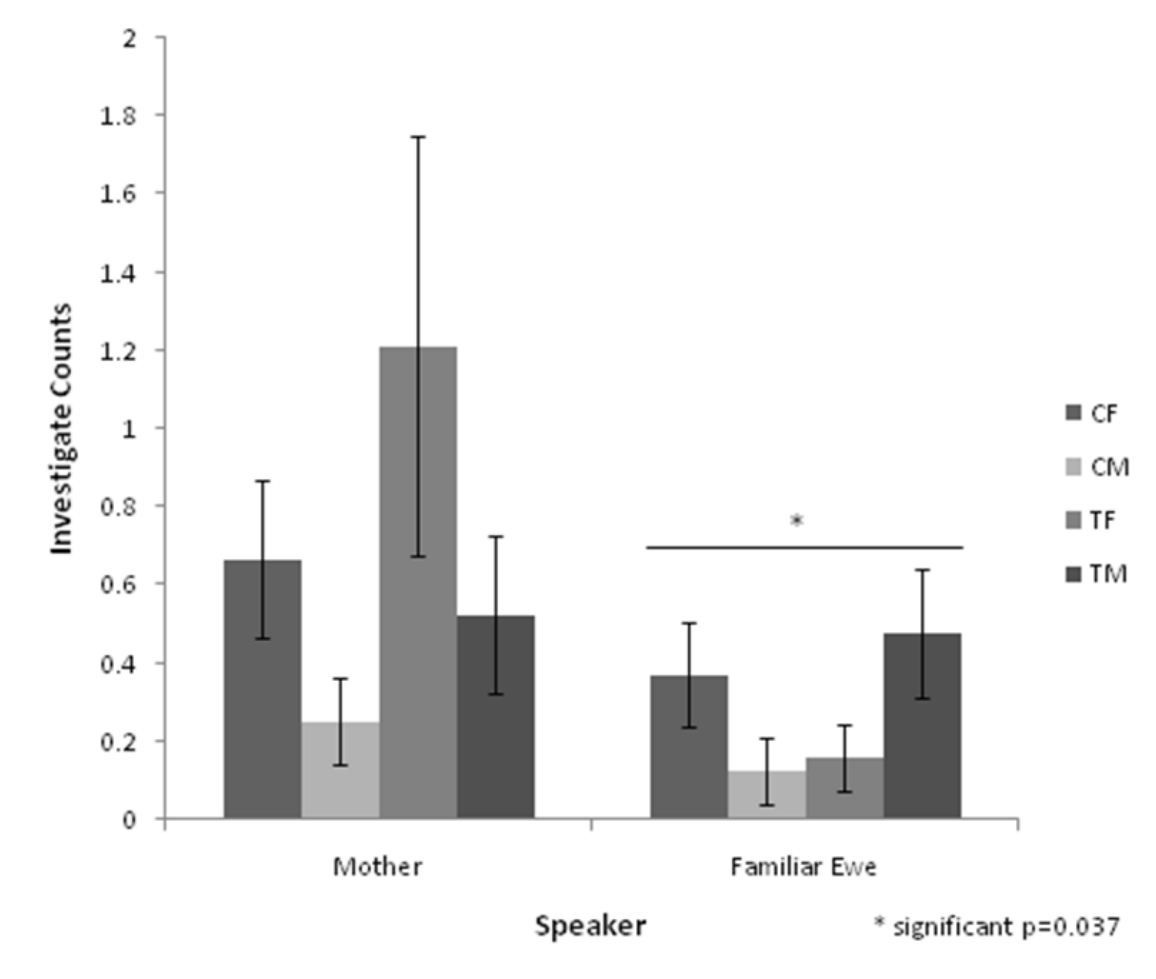


Figure 8.

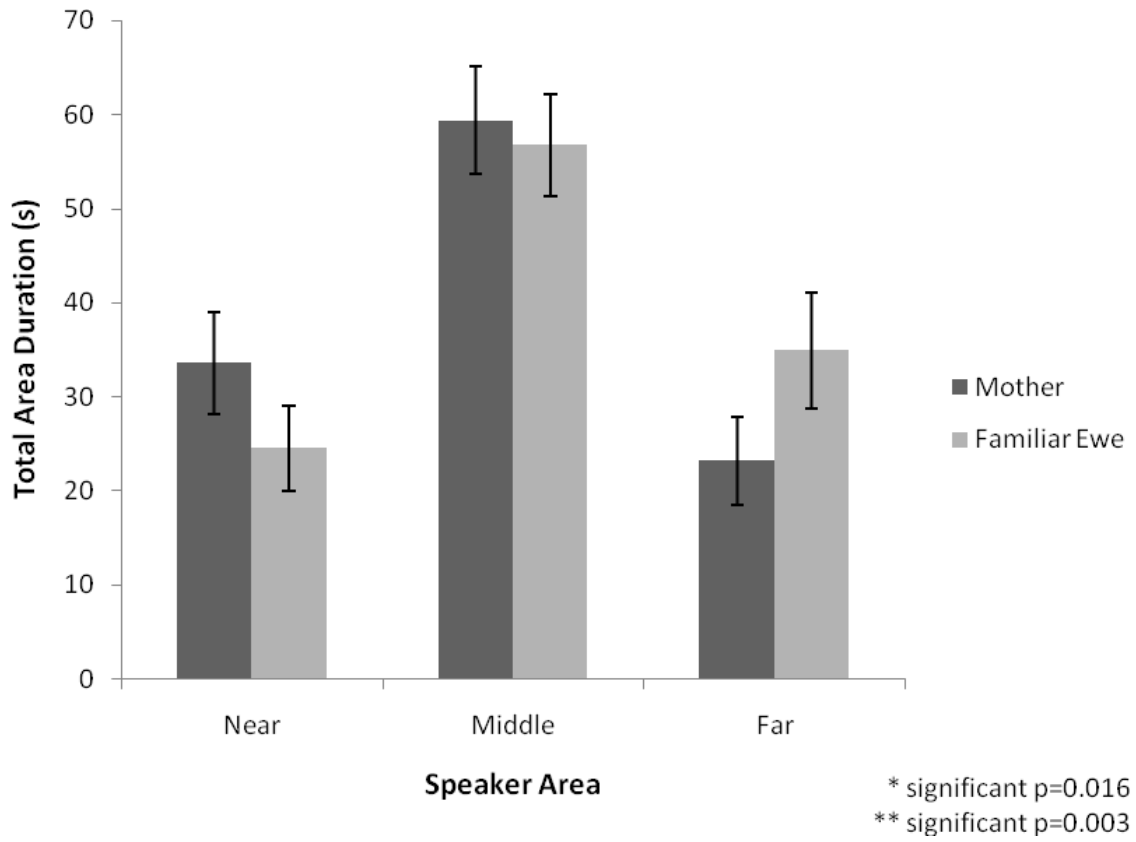


Figure 9.

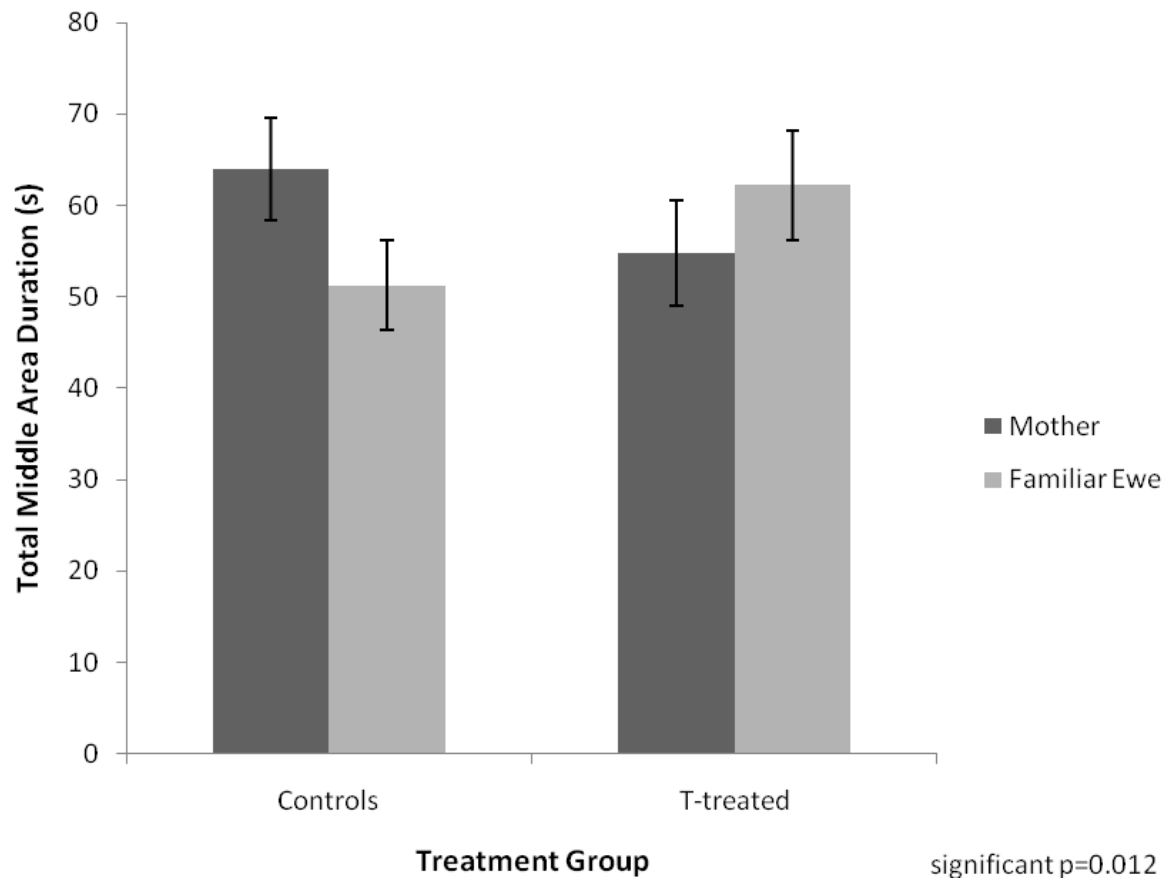


Figure 10.

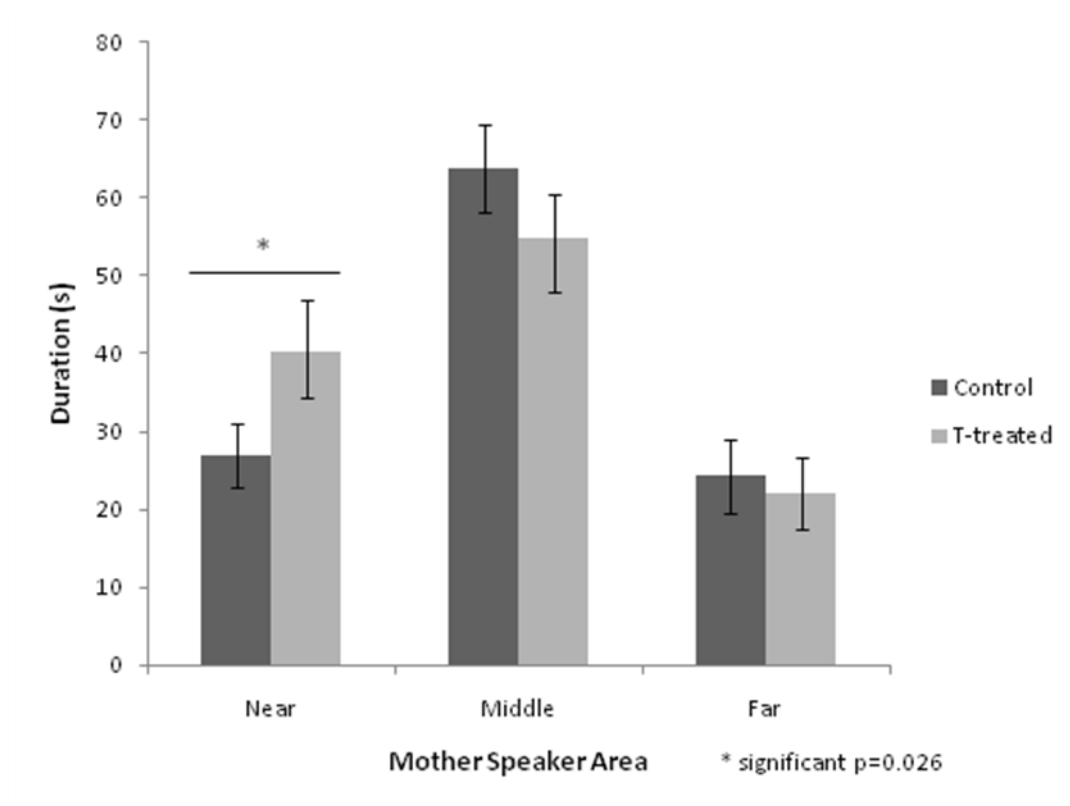


Figure 11.

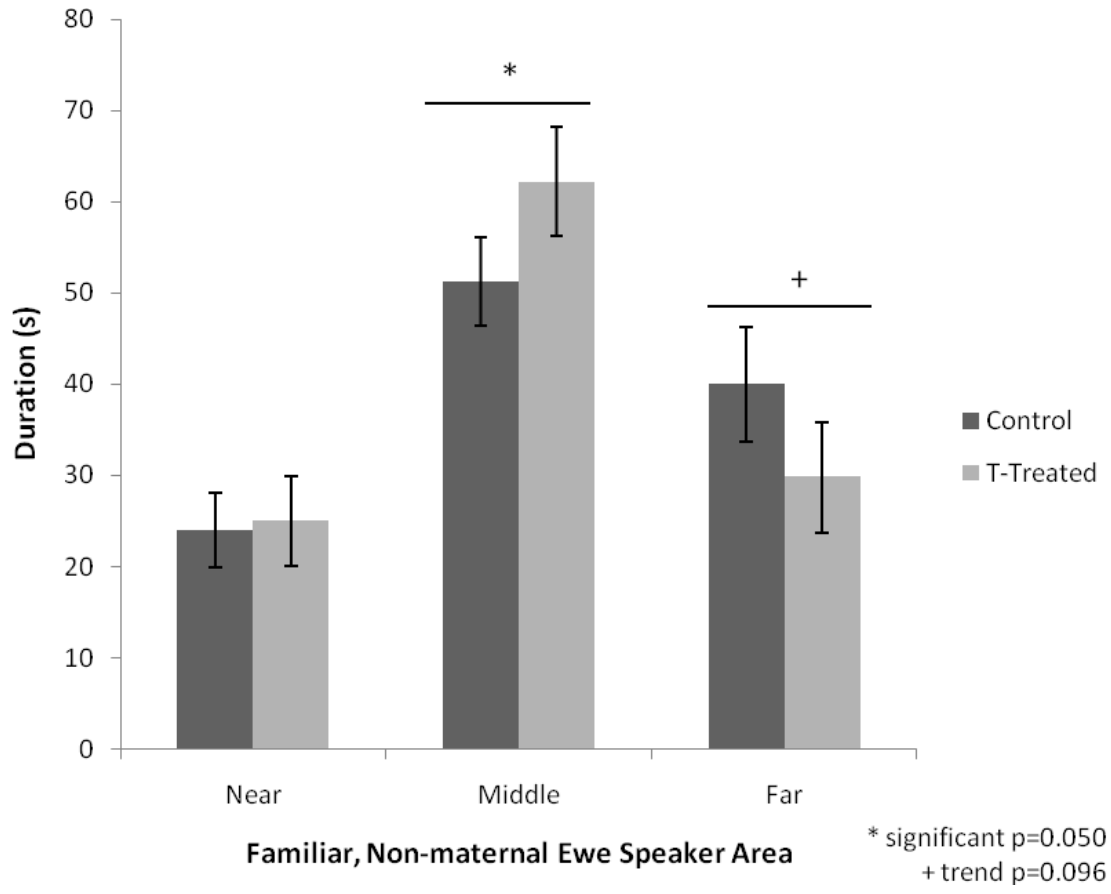


Figure 12.

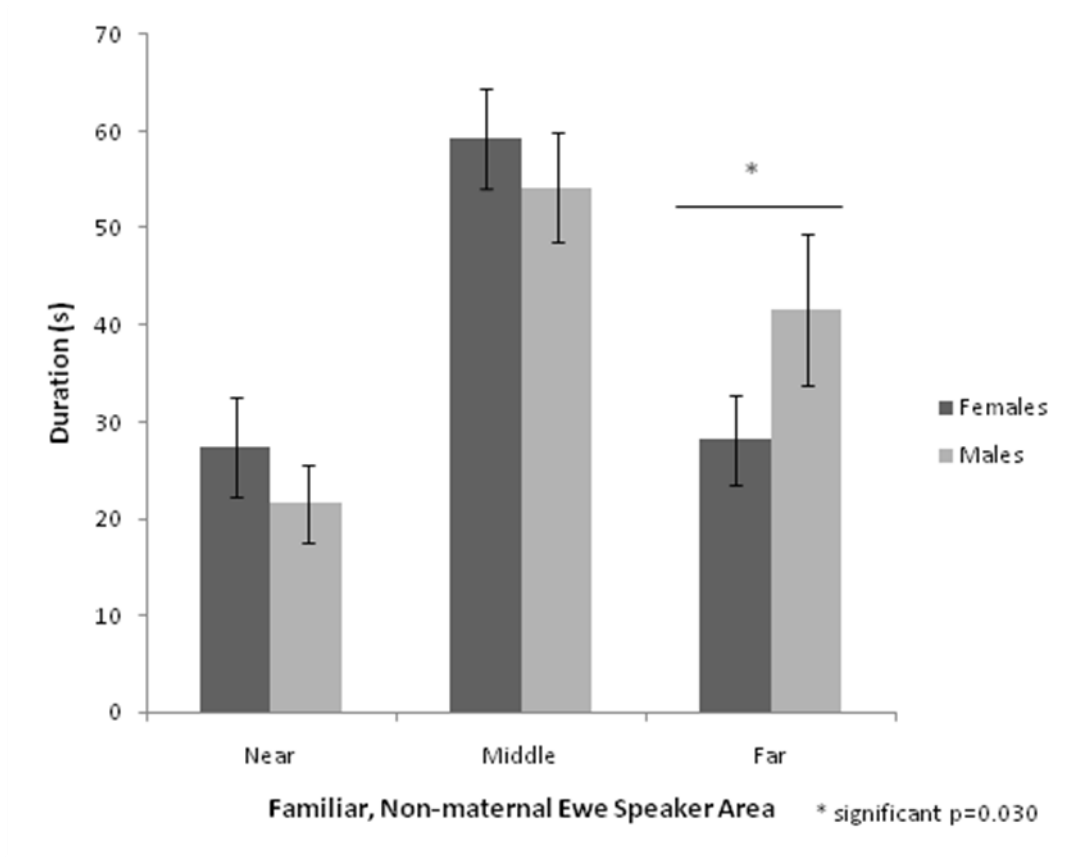


Figure 13.

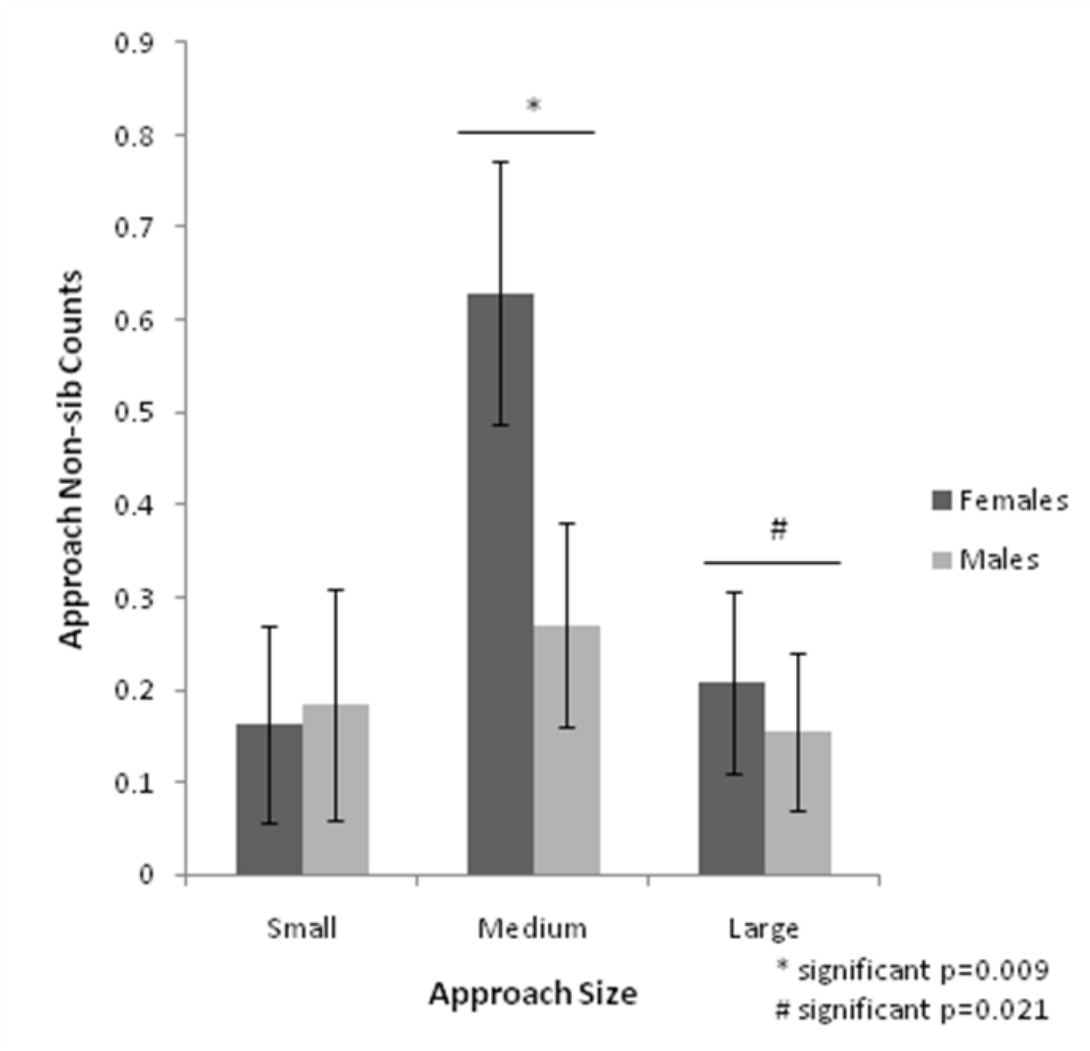




Figure 14.

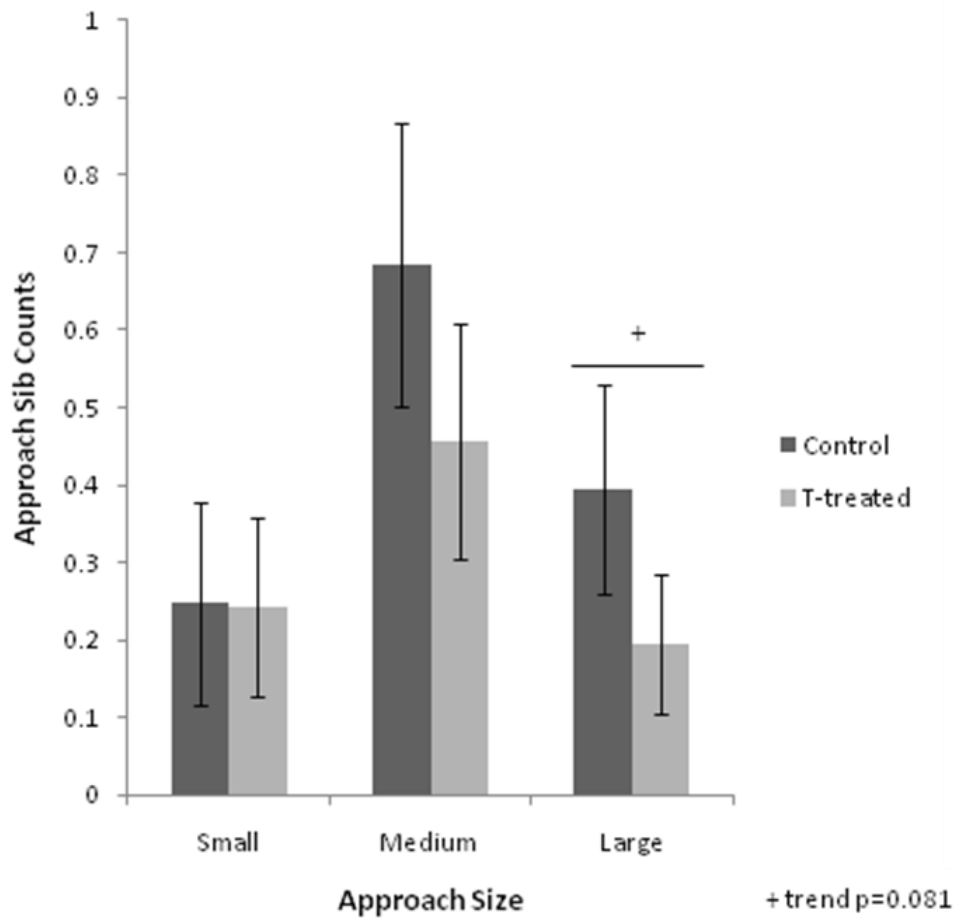


Figure 15.

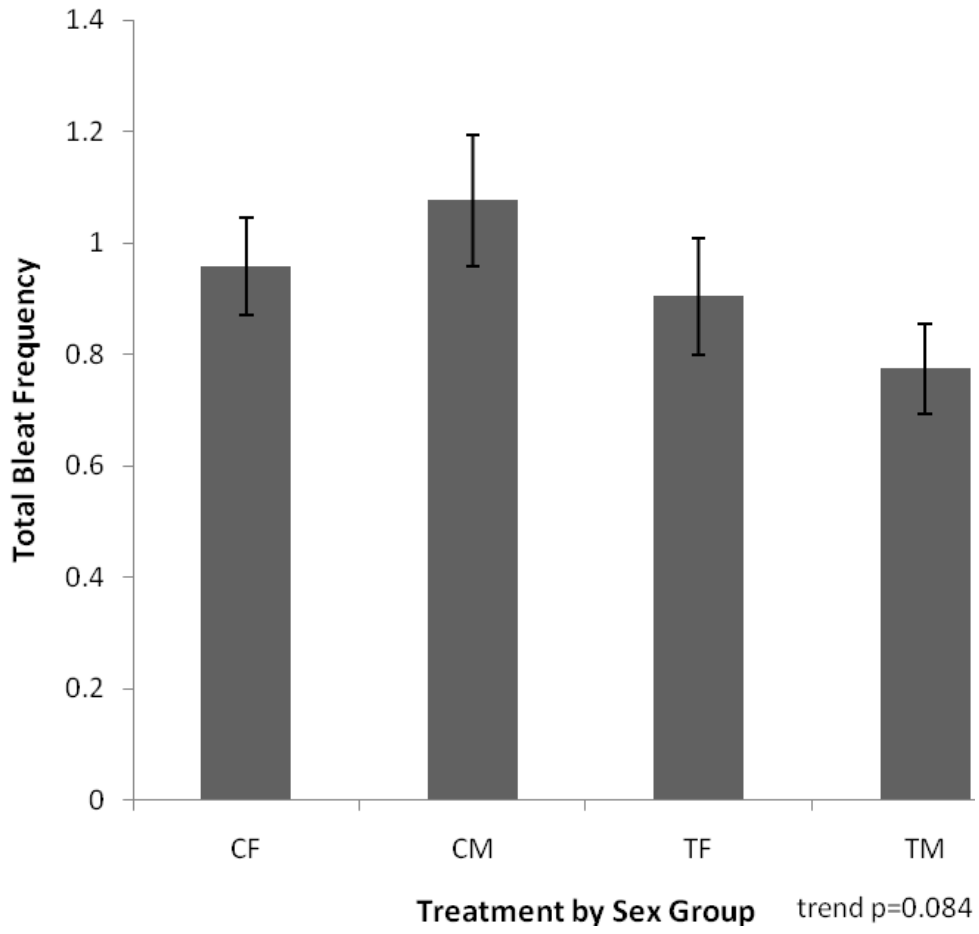


Figure 16.

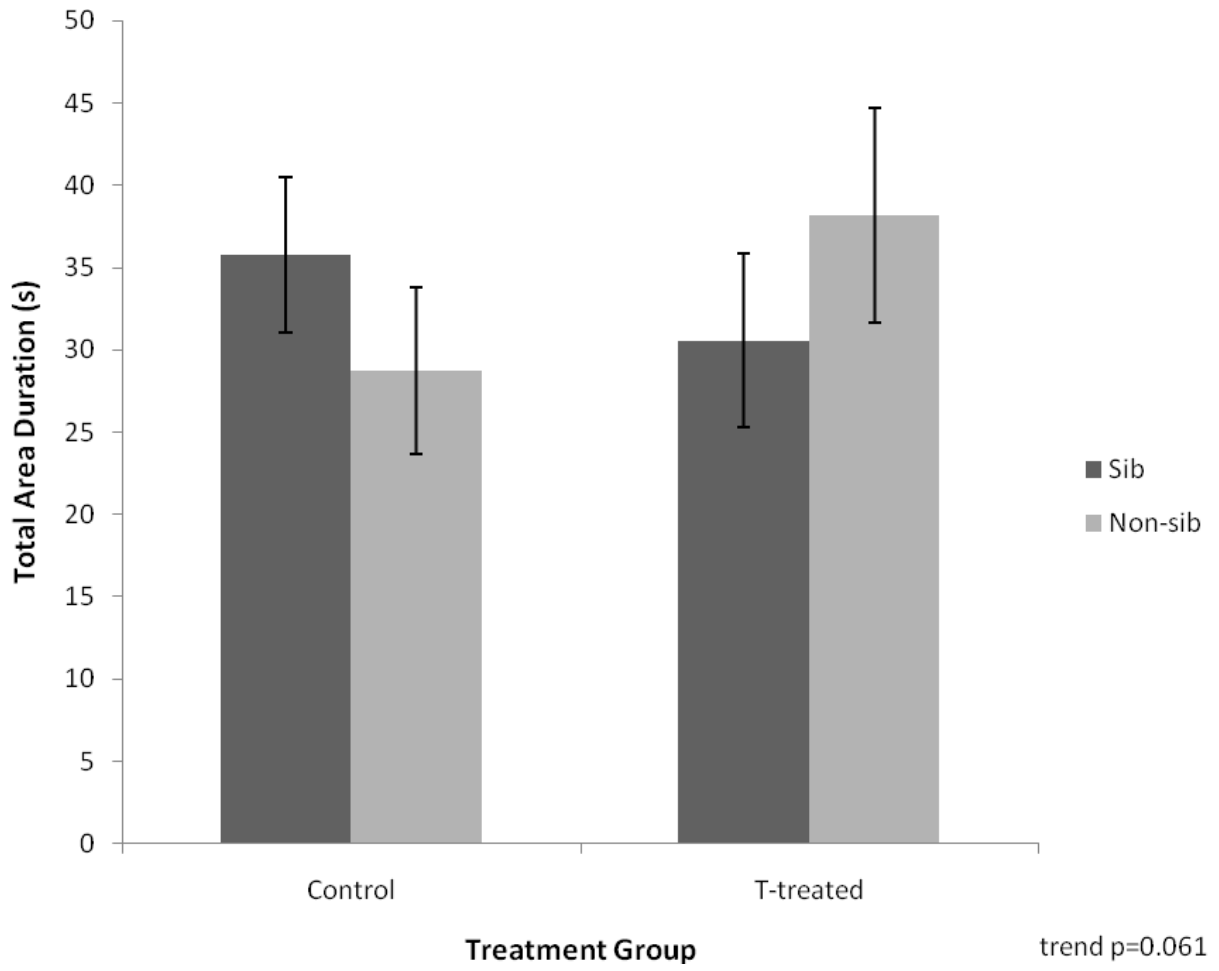


Figure 17.

