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Exploring the Impact of Olfaction on Short-Term and Long-Term Maternal Recognition in Peromyscus californicus

Mariah F. Conley
Seattle Pacific University

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Mariah Conley

Faculty Advisor, Janet Bester-Meredith

Second Reader, Baine Craft

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Abstract

Previous studies have established a connection between social behavior and olfaction, in that as anosmia causes a decrease in perception of social cues, social behavior itself decreases. Studies investigating maternal behavior specifically have focused on foster care, in which the behaviors formed during parturition are conserved and displayed with unrelated pups. The combination of long-term retention of maternal behavior, maternal recognition, and olfaction has yet to be explored. In this study, I induced anosmia in *Peromyscus californicus*, a monogamous, biparental species, and analyzed their behavior with their own pups and with foreign pups in the days after birth, as well as in the weeks after weaning. I concluded that mothers retaining their sense of smell showed a slight preference for their own pup compared to a foreign pup—more so than anosmic mothers. This trend was consistent regardless of the day of testing. Therefore, anosmia, but not time, impaired maternal recognition of offspring.

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As a biparental and monogamous species, *Peromyscus californicus* is a model organism for studying social and parental behavior. One aspect of this behavior is maternal recognition, the ability of a maternal mouse to differentiate between her own pup and a foreign pup. While olfaction is the most prevalent sense in rodents—and a prominent sense in humans as well—the combination of olfaction and maternal recognition have yet to be studied in the California mouse. In other animals, including rats and African striped mice, damaging olfactory mechanisms impacts maternal behavior, but whether that behavior is consistent across time is unknown (Beach et al., 1956; Fleming & Rosenblatt, 1974; Pillay, 2000). Long-term preference studies with California mice and other monogamous species have focused more on sexual fidelity (Gubernick & Nordby, 1993). In this study, I adapted methods from previous maternal behavior and mate preference studies, to investigate the effect of anosmia on maternal recognition in the first months after the birth of pups.

Maternal Memory and Maternal Recognition

Maternal memory is defined as the retention of maternal behavior, which may include behaviors such as huddling, sniffing, nursing, and retrieving. Maternal recognition is discrimination between a maternal mouse's own pup and a foreign pup, such that maternal behavior is performed toward her own pup, or her own litter is given priority over others. These concepts have previously been explored in the way primiparous female rats respond to foster pups (Nephew & Bridges, 2009; Scanlan et al., 2006). Studies investigating longer-term maternal behavior have also focused on foster care. To investigate the importance of parturition on retention of maternal care, primigravid rats who responded maternally following a caesarean

section were presented with test pups. Re-exposure to those pups 25 days later resulted in maternal behavior within five days, with similar results in primiparous rats (Bridges, 1977). This suggests that exposure to pups following the pregnancy is important for establishing maternal behaviors, but that parturition itself may not be necessary (Bridges, 1977). These studies tend to concentrate on development of maternal tendencies during parturition (Bridges, 1975; Bridges, 1977). However, the mother's ability to recognize her own pups in the long-term has received less attention. While the concern of infanticide (Mann et al., 1983) necessitates certain protective measures in pairing maternal mice with both their own and foreign pups, the sustainability of maternal behavior concerning a mother's own pups is worth exploring. In addition, little is known about the role of maternal recognition in species with more complex social systems that include biparental care.

Peromyscus californicus

In comparison to rodents like laboratory rats and mice, *P. californicus* shows a parental care system that is more similar to that of humans: the California mouse is a biparental, monogamous species (Ribble et al., 2003). The fidelity displayed by *P. californicus* is rare in mammals, as only 3 to 10% of mammals exhibit social monogamy, let alone sexual monogamy (Morell et al., 1998). Because humans also exhibit biparental and pair-bonding behavior, studying a species with these commonalities will allow findings to be more significant for future human studies.

Male and female *P. californicus* are rarely spontaneously parental as virgins, but biparental behavior begins at around the birth of their young (Lonstein et al., 2000). In families observed until 31 days postpartum, fathers and mothers both build the nest, and they spend about the same amount of time in the nest (Gubernick & Alberts, 1987). They spend large amounts of

time in the nest simultaneously, but they also spend similar amounts of time covering for the absence of their partner in the nest. Mothers and fathers also spend similar amounts of time in contact with the pups, while fathers spend more time licking pups, and only mothers exhibit nursing. In the first 31 days of a pup's lifetime, the amount of time the pup is left alone in the nest gradually increases (Gubernick & Alberts, 1987). *P. californicus* mothers share similarities with rat mothers in the realm of water recycling. Maternal anogenital licking, followed by ingestion of pup urine, serves to recycle water and electrolytes between the dam and the pup (Gubernick & Alberts, 1987).

P. californicus is the largest species of its genus, with a tail longer than its head and body (Merritt, 1978). Generally, its color is a variation of gray-brown, depending on where in the wild it is found. It is distributed in the southwestern regions of North America from the Baja California peninsula to the San Francisco Bay (Merritt, 1978). It is also nocturnal, with increases in activity immediately before dawn (Jess, 2000). Both males and females display sexual fidelity, and they have a short gestation period of 30 to 33 days. Their breeding season is usually from March to September, and their average litter size is two, making them ideal for studying in a laboratory setting (Jess, 2000).

The investment that both parents make in their young sets *P. californicus* apart compared to other rodents often used in laboratory research (Lonstein et al., 2000). Norway rats, *Rattus norvegicus*, are frequently studied for their sex differences in parenting, as the lactating dams are the sole providers of care for their offspring (Lonstein et al., 2000). *Mus musculus* are also popular in parental behavior studies, but they show more variability in responsiveness depending on their specific strains. Some strains are biparental, while others show males sharing nests with females but not participating in parenting (Lonstein et al., 2000). By adulthood, most female *M.*

musculus are spontaneously maternal, while males may be infanticidal. In meadow voles, *Microtus pennslvanicus,* only lactating dams care for their young (Lonstein et al., 2000). Prairie voles, *M. ochrogaster,* are socially monogamous and biparental: kyphosis (a posture of nursing and huddling) is even expressed by both males and females (Lonstein et al., 2000). Several hamster species display infanticidal tendencies, and hamster pups are often reared only by their dams (*Mesocricetus auratus, Phodopus sungorus*). That California mice are biparental makes them an ideal model species for parental behavior studies.

Olfaction and Maternal Recognition

While the link between olfaction and maternal behavior has been established in a variety of species, less is known about the effects of olfaction on a mother's ability to recognize her pups. When anosmia is induced in rats, behavioral changes result: the usually aversive behavior of virgin females toward pups is eliminated, and the females adopt maternal behavior (Fleming & Rosenblatt, 1974). Additionally, in the hamster, infanticidal subjects demonstrate pup-carrying behaviors after their vomeronasal nerves are cut (Marques, 1979). Anosmia induced by zinc-sulfate results in the majority of female mice eating their offspring as well (Seegal & Denenberg, 1974).

One particularly interesting finding is that female rats demonstrate discriminatory behavior, retrieving their own pups before alien pups (Beach et al., 1956). Presented with a group including five of her own pups and one foreign pup, six of seven females initially rejected the foreign pup. When the group consisted of five foreign pups and one of her own, the females investigated and rejected each of the alien young, found and retrieved their own, and then returned for the five aliens (Beach et al., 1956). After the olfactory bulbs of previously discriminatory females were removed, they displayed unimpaired retrieving ability, but did not

discriminate (Beach et al., 1956). However, olfactory cues may be more important for the California mouse, a species where females and males will commit infanticide against unfamiliar pups.

The onset of responsiveness to olfactory cues and subsequent maternal behavior usually occurs at parturition (Lévy et al., 2004). Female rats, during the peripartum period, tend to prefer bedding soiled by pups to clean bedding, while virgins and pregnant females do not show that preference (Lévy et al., 2004). Virgin rats treated with hormones that mimicked parturitional changes preferred pup-related odors (Fleming et al., 1989). Even in humans, new mothers given a choice between infant odors and control odors will give significantly more positive ratings to the infant odors (Fleming et al., 1993).

In defining what constitutes olfactory investigation, previous studies have cited direct and active exploration, especially nosing and sniffing of head and anogenital regions, as well as pursuit (Bielsky et al., 2004). Displaying investigatory behavior less over time toward a juvenile would constitute social recognition in both rats (Nephew & Bridges, 2008) and mice (Bielsky et al., 2004). Recognition is implied because mice are prone to investigate less upon subsequent exposure to the same individual (Bielsky et al., 2004). However, while these studies imply behavioral patterns for social recognition, they do not explicitly apply to maternal recognition, because they investigated the behavior of males toward novel ovariectomized females (Bielsky et al., 2004). Still, there is chemosensory signaling at play in maternal behavior. Females shown to display impaired detection of odorants also demonstrated reduced pup retrieval behavior and reduced maternal aggression (Wang et al., 2011). Furthermore, the pups of those impaired mice appeared to be scattered throughout the cage, suggesting decreased maternal behavior. When

tested, the unimpaired mice retrieved the pups, and also exhibited licking, crouching, and nest building (Wang et al., 2011).

Olfaction and Recognition in Humans

Human mothers are able to use olfaction to identify garments worn by their infants days after giving birth (Porter et al., 1983). For human babies, nursing is motivated partly by maternal olfactory signals (Cernoch & Porter, 1985; Porter & Winberg, 1999). Humans use olfaction not just in recognition of parent or offspring, but also during recognition of siblings and acquaintances. In a study examining recognition of kin, familiar non-kin, and strangers, blindfolded participants were presented with t-shirts worn for two consecutive nights by their subjects (Weisfeld et al., 2003). For the total combined stimulus categories, the participants were able to accurately name the source of the shirt's odor 63% of the time. Stimuli from kin were least likely to be confused with non-kin. Stimuli from subjects of the same sex were also more likely to be misidentified than stimuli from opposite-sex subjects (71% and 29% of total errors, respectively). In general, the authors concluded that participants were able to discriminate between related family members, and could also recognize friends and spouses (Weisfeld et al., 2003).

Another study sought to discover the degree to which mothers differentiate between children and stepchildren (Weisfeld et al., 2003). Again using shirts worn on consecutive nights as odor stimuli, mothers could correctly identify their biological children's odors the vast majority of the time. However, that level of recognition did not apply to their stepchildren. Furthermore, by the same methods, children could identify their biological siblings, but not their stepsiblings or half-siblings. According to Porter (1999), the use of olfaction to differentially recognize individuals between social categories is perhaps preferable to other senses, given its

consistency and transferability. Odor, and the perception of odor, does not change with the time of day or environment, which may make it preferable to vision (Porter 1999). Furthermore, chemicals responsible for odor can be transferred from the subject onto an object and can be preserved there—a quality utilized by Weisfeld et al. Exploring olfaction in California mice, therefore, may produce results applicable to other biparental mammals, such as humans.

Logistical Complications with Measuring Maternal Recognition

Both human and rodent studies suggest that maternal recognition is apparent—if not developed—soon after birth. Therefore, to elucidate the mechanism behind maternal memory, the events surrounding parturition provide a compelling place to start (Byrnes et al., 2002). As evidence of maternal behavior, researchers look for retrieving, grouping, and crouching over pups (Nephew et al., 2008). However, observing for maternal behavior could lead to confusion if the subject is exposed to both her own pup and a foreign pup, as there are not clear observable differences between investigatory behavior and maternal behavior when analyzing video. For example, a mother moving toward her pup to retrieve it constitutes maternal behavior, but pursuit is also an example of investigatory behavior (Bielsky et al., 2004). Therefore, it may not be possible to distinguish a subject exhibiting maternal behavior toward her own pup and investigatory behavior toward a novel pup.

Drawing an analogy between a mother preferring her own pup and a female preferring her mate may prove useful in measuring maternal recognition. In both cases, the female in question has a longer-term relationship with the other subject. When presented with unfamiliar estrous virgin females, male mice preserve their sexual fidelity, even in the absence of their partner (Gubernick & Nordby, 1993). Females exhibit similar behavior, in which fidelity can be seen in not only copulation, but proximity and quiet contact. Moreover, with their mates present,

females may attack a stranger (Gubernick & Nordby, 1993). In prairie voles as well, partner preference is indicated by copulation, but also selective contact and affiliation with the partner over a stranger (Young et al., 2011). This finding could carry over to maternal behavior, if a female recognizing her pup spends more time with it than a new pup, and demonstrates fewer signs of aggression toward it. Most males, as well, ignore or attack unfamiliar pups before becoming parental with their own (Gubernick et al., 1994).

When a female African striped mouse, *Rhabdomys pumilio,* is not offered a choice, but rather has her own pups exchanged for foster pups, she transfers maternal behavior onto the foster pups (Pillay, 2000). Exchanges earlier in pup development yielded similar growth rates among fostered and non-fostered pups, but exchanges later (10 days after birth) yielded reduced growth rates for foster pups. In exchanges after day 12, females exhibited aggression toward foster pups (Pillay, 2000). Pups fostered and returned to their mothers later were accepted if they were young (less than 10 days old), but by the time the pups were 14 to 16 days old, they were rejected (Pillay, 2000). Given that pups switch from suckling to eating solid food at around 10 days old, female mice may be displaying aggression toward the diet change, and not be able to distinguish between fostered and non-fostered pups (Pillay, 2000). While this study provides important information about retention of maternal behavior, it uses foster pups rather than the mother's own pups, and it also does not explore the role that olfaction plays in maternal behavior.

Additionally, different species respond differently to foster pups. Cox et al. used mixed litters with their foster dams, in an experiment investigating the effects of gestational bisphenol A exposure on mouse behavior. They included up to two of the dam's own pups with each litter to minimize stress and infanticide (Cox et al., 2011). With *P. californicus,* precautionary steps

must also be taken. *P. californicus* mothers do not readily accept young pups, as they tend to discriminate using olfactory cues (Bester-Meredith, 2016). In both cross-fostering and in-fostering contexts, pups are wiped clean with cotton balls soaked in water, and dipped in their foster parents' soiled bedding (Bester-Meredith et al., 2001).

This study seeks to measure maternal recognition by imposing a choice test. To avoid infanticide, the maternal recognition testing cage uses dividers to separate the pups from the maternal subject. Because the maternal mice are not allowed physical contact with the pups, behaviors such as retrieving, huddling, and nursing are impossible. Instead, proximity is used to measure preference, and preference constitutes maternal recognition, just as it indicates fidelity for mate preference studies. Mesh panels in the dividers allow sensory cues to pass, such that the subjects with olfaction intact can still smell the pups on either side. If, like the African striped mice, California mice exhibit changes in maternal recognition over time, this will be detected by conducting tests that span the first two months of the pup's life. Because olfaction is the primary sense used by California mice, I predicted that mice retaining their sense of smell would discriminate between their own pup and a foreign pup, as evidenced by preference of the own pup's side in the testing arrangement. I hypothesized that anosmic mice would not demonstrate the same degree of preference, if any. Based on the results from Pillay et al., I did not think that subjects would demonstrate maternal recognition after the pups were weaned, whether or not they were anosmic.

Methods

Housing

California mice were housed in the Seattle Pacific University animal care facility, where they were monitored daily by facility staff and cared for under the Guide for the Care and Use of

Laboratory Animals, set by the National Research Council (2011). Two groups of six first-time mothers were selected from the breeding colony and housed in the facility, each in her own separate polycarbonate cage. The room was kept at a temperature of approximately 23 ºC, under a 14:10 hour day: night cycle, and food and water levels were monitored daily. Sexually naïve female mice were paired with one or two other females of similar age until they were paired for mating, living in standard (45 x 24 x 14 cm^3) cages with wire mesh lids and aspen shavings, as well as enrichment (Envirodry). Each of the eleven total females was paired with an unrelated male, and those pairs were housed in their own separate cages through the birth and weaning of pups.

A set of observation cages was made for the females and their pups as well. The observation cages used for olfaction tests were made of polycarbonate (52 x 29 x 30 cm^3) and had two distinct chambers separated by a transparent wall, with two round holes joining the chambers, positioned above the floor of the cage. This arrangement was ideal for monitoring maternal behavior and recognition. A different kind of observation cage (three chambers of 30.5 x 30.5 x 30.5 cm^3) based on one previously used (Gleason et al., 2012) for the maternal recognition testing had one main chamber and two side chambers. Rectangular holes joining the chambers were present (11.5 x 11.5 cm), but covered in mesh, such that the mice in the side chambers could communicate with their adjacent chamber without physically entering.

Olfaction

Olfaction tests have been used historically to assess behavior of mice (Yang & Crawley, 2009). The morning after eleven female mice were paired with the males, each female underwent intranasal injection procedures. A random six females received injections of 33 mM zinc gluconate, 50 µL into each nostril (Duncan-Lewis et al., 2011), and the other five received sterile

H_2O. Before and after usage of the needles, they were sterilized with heat using the Germinator. This procedure was repeated every 12 to 16 days to maintain anosmia.

The efficacy of zinc gluconate for inducing anosmia has been demonstrated previously (Slotnick et al., 2007), but was confirmed with pre-tests and post-tests. One day before the injections were given, two trials of a hidden apple test were conducted for each female. In this test, the observation cages contained a layer of aspen bedding on the cage floor, and an apple slice was buried under the bedding in a designated location. To familiarize the mice with the apple slices, an open apple test was conducted before the hidden apple tests.

On the floor of the observation room, two cages at a time were placed side by side, with an opaque barrier separating the cages to prevent the mice in each cage from seeing one another. The night before testing, food was removed from the subjects' cages to induce an overnight fast. Each mouse was gently removed from her housing cage, dropped into the cage, and filmed for ten minutes. Food was replaced in the housing cages after the conclusion of the tests each day. After the injections were administered, with one hour between the last injection and the first post-test, the hidden apple test was repeated with all the mice. Again, they were filmed for ten min.

A series of bedding tests also confirmed the success of anosmia-inducing injections (Table 1). These used the same cages as the apple tests, but rather than hiding an apple, four weigh boats of bedding were placed in the four corners of the cage. In the clean bedding pre-test, each boat contained a handful of clean bedding and Envirodry. For the soiled bedding tests, weigh boats were labeled A, B, C, and D. Boat A held soiled bedding from the test mouse's own cage, which was collected and placed in the bin immediately prior to testing. Boat B held soiled bedding from male cages, Boat C held clean bedding, and Boat D held soiled bedding from

female cages. Soiled bedding collected prior to the test date was gathered from several different cages, stored in a freezer, and thawed before the test itself. One round of a clean bedding test and two rounds of soiled bedding tests were conducted the day before intranasal injections, and one soiled bedding test was conducted after injections (Table 1). Mice were paired at the end of olfaction testing.

Maternal Recognition Testing

After the pups were born, on days two, six, and ten, both the control and the anosmic mice were observed for fifteen min (Table 1). One newborn pup from the subject and a pup from a different maternal female were placed in an observation cage. The mothers did not have previous familiarity with the observation cages, in order to prevent a sense of security that could have made them territorial and increase aggression unnecessarily. The cage was clean, with new bedding, but free of food and water to avoid distractions. As used previously in sexual fidelity procedures (Gubernick & Nordby., 1993), the stimulus mice (the pups) were placed in randomized side chambers. Polycarbonate barriers with square holes were placed between the side chambers and the main chamber. However, mesh panels over the holes allowed scent to pass though the barrier without allowing physical contact. Immediately after the pups were secured, the mother was placed in the center chamber. After testing, the pups and dams were returned to their respective cages.

These observations in the days after the pup's birth constituted short-term maternal recognition. Long-term maternal recognition testing occurred after the pups had been weaned, on days 2, 10, and 30. Again, the newly weaned pup from the subject and the newly weaned pup from a different maternal female were placed in randomized sides of the observation cage at the same time. They were also observed for 15 min (Table 1).

Analysis/Statistics

Each video was analyzed by two separate observers, whose training consisted of analyzing sample videos until they reached consistency between trials and between each other. The observers recorded the latency to find the apple, which was indicated by prolonged sniffing, pawing at, or picking up and eating the apple slice. The observers also recorded the duration of time sniffing each bedding boat and the frequency of dumping, moving, digging through, laying in, and returning to each boat. For the hidden apple videos, if the mice injected with zinc gluconate found the apple with a significantly increased latency than those treated with sterile H_2O, they were considered anosmic. Furthermore, a lack of preference for a particular kind of bedding in the soiled bedding tests indicated anosmia as well.

In the maternal recognition testing, as the mother was not physically able to enter the side chambers, sniffing or exploring immediately by the mesh barriers in the partition constituted sniffing or exploring the pup in that specific chamber. Therefore, in analysis of their interactions, duration was measured for sniffing or digging by each side chamber. Furthermore, the frequency of returns to each side chamber was counted. More time with the subject's own pup constituted maternal recognition.

A paired-sample *t*-test was used to compare the zinc gluconate latencies between the second hidden apple pre-test and the hidden apple post-test. A repeated measures ANOVA compared the sterile water and zinc gluconate groups for the bedding tests. Statistical significance was determined at $p < 0.05$. With the maternal recognition testing, the sample sizes were not high enough to conduct any meaningful statistical analyses, but trends were elucidated from graphs of the data.

Results

Olfaction

In the apple tests, general trends are discernible between the two groups, even though none of the results were statistically significant. For example, in the open apple test, mice in the zinc gluconate group demonstrated a higher latency to find the apple than mice in the sterile water group (Figure 1). Both groups decreased their latency between the open apple test and the first hidden apple test, although the mice treated with sterile water showed a sharper decrease than the zinc gluconate mice. An hour after both groups were given intranasal injections, the hidden apple post-tests took place. Here, the zinc gluconate mice did not take longer to find the apple than the mice treated with sterile water. Although the zinc gluconate mice demonstrated their highest latency in the post-test (Figure 1), it was not significantly higher than that of their previous hidden apple test [df = 10, *power* ($1\text{-}\beta$) = .41, p = 0.34, d = .43].

In the first bedding test, during which all four bins held clean bedding, neither the sterile water nor zinc gluconate groups displayed any significant difference in their behaviors toward the bins [$F(1, 3) = 3.40$, $p > 0.05$, $d = 0.27$]. The behaviors measured included sniffing, digging, returns, sitting in, dumping, and moving the bins. There were also no significant interactions between the behaviors measured and the groups (sterile water or zinc gluconate) [$F(1, 3) = 1.06$, $p > 0.05$, $d = 0.11$].

In the soiled bedding pre-test, specific behaviors were directed at particular bedding bins (Figures 2-7). Sterile water and zinc gluconate groups differed in their digging behaviors between the four types of bedding, but did not differ in any other behavior [$F(1, 1.52) = 6.36$, $p = 0.03$, $d = 0.41$]. Mice also appeared to show preferences for different types of odors, as evidenced by differences in sniffing [$F(1, 1.65) = 4.32$, $p = 0.04$, $d = 0.32$], returns [$F(1, 3) = 3.70$, $p = 0.02$, $d = 0.29$], and dumping [$F(1, 3) = 3.18$, $p = 0.04$, $d = 0.26$], depending on the

contents of the bins. Although there were no other statistically significant differences, and small sample sizes did not allow for post-hoc tests, the frequencies of each measured behavior seem to differ by bin for the mice treated with sterile water. For example, both the sterile water and zinc gluconate mice appear to sniff soiled male and soiled female bedding with a longer duration than their own bedding or clean bedding (Figure 2). The mice treated with sterile water give the impression that they sniff soiled bedding longer than the zinc gluconate mice, but the two groups exhibit similar behavior for the other two bedding types. The same trend, both within and between groups, appears for dumping behavior as well.

The slight differences in sniffing and dumping between bedding types exhibited in the soiled bedding pre-test are not apparent in the soiled bedding post-test (Figures 2-7). However, similar to the soiled bedding pre-test, mice appeared to return to the four bedding types with differing frequencies [$F(1, 3) = 4.768$, $p = 0.024$, $d = 0.35$]. Although this cannot be proven statistically, the mice treated with sterile water appear to exhibit the highest frequency for their own bedding compared to the others. By contrast, the zinc gluconate mice do not demonstrate any preference between the four bedding bins. In general, between soiled bedding pre-tests and post-tests, the zinc gluconate mice seem to spend less time sniffing overall after intranasal injections (Figure 2). Sample sizes for the sterile water and zinc gluconate groups were five and six, respectively.

Maternal Recognition

Mice in both the sterile water and zinc gluconate groups spent the vast majority of their time on either side of the central chamber in the maternal recognition cage (the proportions ranged from 73% to 98%, Figure 8). That value was then designated as *exploratory time*, and the proportion of exploratory time spent on the side of the subject's own pup was measured for each

behavior. In the sterile water and zinc gluconate groups, sample sizes were four and three, respectively.

In terms of total behavior (including both active and inactive behaviors), the mice treated with sterile water spent over 50% of their exploratory time with their own pup in the days after birth (Figure 8). After weaning, that behavior was maintained. The zinc gluconate group spent a lower proportion of their time with their own pup, in both post-birth and post-weaning tests. Generally, the mice treated with sterile water spent a greater proportion of their time with their own pup than the zinc gluconate mice.

Looking specifically at touching behaviors (Table 1), on days 2, 6, and 10 after the birth of the pups, sterile water mothers spent more time touching the side of their own pup than touching the side of their foreign pup (Figure 9). This difference was not apparent in the zinc gluconate mothers, who spent less than 50% of their time touching their own pup's side. On days 2, 10, and 30 after the pups were weaned, sterile water mothers still spent most of their total touching time on the side of their own pup, and zinc gluconate mothers spent less. However, the time mice treated with sterile water spent with their own pups decreased between the post-birth and the post-weaning tests. The zinc gluconate mice, after their pups were weaned, yielded inconsistent results. While their time spent touching their own pups generally increased between the short-term and long-term tests, it did not surpass the mice treated with sterile water.

With digging behaviors, mice treated with sterile water again spent over half of total digging time on the side of their own pup, and zinc gluconate mice did not (Figure 10). Unlike touching behaviors, but like total behaviors, this trend was apparent in both the short-term and long-term tests. In fact, the digging differences between mice treated with sterile water and zinc gluconate mice increased toward the end of testing. The climbing results resembled those for

digging, in that the behaviors between the two groups differed more in the long-term tests than in the short-term tests (Figure 10-11). However, like the touching results, they also exhibit some inconsistency. On day 6 after birth, the zinc gluconate mice climbed by their own pups more than the mice treated with sterile water did. This overlap only occurs in one other instance, on day 2 after weaning for the touching behavior.

Combining touching, digging, and climbing provides a broader picture of all *active* behaviors (Figure 12). Here, mice treated with sterile water spent the vast majority of their total active behavior time exhibiting that behavior toward their own pups. However, zinc gluconate mice spent just under half of their total active behavior time with their own pups.

Discussion

In general, despite the lack of statistical significance, the trends of the data suggest that the sterile water and zinc gluconate injections yielded the desired effects, inducing anosmia in the zinc gluconate mice while leaving olfaction intact in mice treated with sterile water. This conclusion is most directly supported by the zinc gluconate group, in their appearance of decreasing latency to find the apple before intranasal injections, and increased latency after injections. The maternal recognition tests, therefore, accurately reflect differences between anosmic and normal maternal mice. Indeed, the mice treated with sterile water appear to demonstrate a preference for their own pups. This preference is consistent between the measured behaviors across time, and is absent from the zinc gluconate group.

Olfaction: Apple Tests

In the apple tests, the zinc gluconate group demonstrate the desired results. Between the two hidden apple pre-tests (Hidden Apple 1 and Hidden Apple 2 in Figure 1), their latency to find the apple decreased, which demonstrated that their sense of smell was intact, and they were

growing more familiar with the scent of the apple. After intranasal injections, their latency increased, which implies that anosmia was successfully induced. If their sense of smell had been intact after the intranasal injections, I would have expected to see their latency to find the apple decrease, just as it did between the first two hidden apple tests, as well as in previous olfaction testing (Bester-Meredith, 2017). While the *t*-test does not show a significant increase in latency between the last pre-test and the post-test, the power of the test was low. This is most likely due to the small sample size, as zinc gluconate has been shown to induce anosmia in previous studies (Bester-Meredith, 2017; Duncan-Lewis et al., 2011; Slotnick et al., 2007).

One concerning factor in the apple tests was that the pre-test results differed between the sterile water and zinc gluconate groups, even if the results were not statistically significant (Figure 1). As neither group had been injected yet, one would expect that their latency would be roughly the same. However, the group that would be receiving zinc gluconate injections took nearly twice as long to find the open apple as the sterile water group. The difference in latency between the two groups only increased with the first hidden apple test. In the second hidden apple test, the mice treated with sterile water took their longest time yet to find the apple. Theoretically, their latency should decrease as they become more familiar with the scent of the apple. This happened with the zinc gluconate mice, but not the mice treated with sterile water. After the injections, the mice treated with sterile water should have retained their sense of smell. However, the mice treated with sterile water had a greater latency than the zinc gluconate mice in this trial, too. Because the sterile water itself could not have induced anosmia, perhaps it was the stress of the injection process that affected the group differently than the zinc gluconate mice. However, this would not account for the seemingly increased latency between the hidden apple tests. Given that the sample sizes were small, it is difficult to draw any conclusive

generalizations from the data, despite the reliability of using apple tests to measure olfaction (Curtis et al., 2001; Portillo & Paredes, 2004).

Olfaction: Bedding Tests

Based on the clean bedding tests, neither group displayed an initial preference for a location within the cage. Because each bedding bin in this test contained clean bedding from the same source, the mice should have preferred each bin equally in all of the measured behaviors, as suggested by the results. Insignificant results in this case are helpful, because they mean that there was no location bias to consider in the statistical analysis for the rest of the data.

With the soiled bedding pre-tests, the mice treated with sterile water appear to generally prefer bedding boat D, which contained soiled bedding from females (Figures 2-7). This preference for soiled bedding has been demonstrated in previous testing (Bester-Meredith, 2017). Based on the average values reflected in the graphs, mice treated with sterile water spent more time sniffing soiled female bedding than any other type. They also appeared to dig, sit in, dump, and move the female bedding more frequently than the others. These differences are not as visually apparent in the zinc gluconate group, perhaps because the behaviors in general are less frequent. Based on the duration of sniffing and frequency of returns, the zinc gluconate mice appear to prefer the soiled bedding bins (own, male, and female) over the clean bedding bin. However, this trend is not suggested by the other behaviors. As neither group had received injections at this point, I would not expect there to be a difference between sterile water and zinc gluconate mice, especially given the precedent for using odor stimulus preference in olfaction testing (Yang & Crawley, 2009). Based on the small sample size and high standard error values for most measured behaviors, however, insignificant results are not surprising.

After the injections, in the soiled bedding post-tests, the mice treated with sterile water appear to retain preference, but for a different bin than in the pre-tests (Figures 2-7). Based on the graphs, mice treated with sterile water dug in, dumped, and returned to their own soiled bedding most frequently. They also spent more time sniffing their own soiled bedding and male soiled bedding than the other two types. The zinc gluconate mice appear to demonstrate no preference in any behavior for any bin, especially given the wide standard error bars.

While hardly any bedding results were significant, no statistical test had high power. This is likely due to the limited sample size and generally low frequencies of the measured behaviors, which also contributed to high standard error. These issues are perhaps most apparent in the soiled bedding post-test graph for moving. Here, the average frequency of moving in the zinc gluconate group was zero, and in either group, frequency never reached one.

Across the different bedding tests, the most reliable behaviors upon which to base tentative results are likely sniffing and returns. Because there are generally more returns than the other behaviors (returns range from around five to fifteen, while other behaviors rarely pass five), there is more potential for capturing bin preference. Likewise, the average duration measurements for sniffing did not dip below five seconds, and more often ranged from ten seconds to over one minute. With this in mind, looking only at sniffing and returns, there appears to be no difference between the sterile water and zinc gluconate groups in either the clean bedding test or the soiled bedding pre-test, especially given how the error bars overlap within each bedding bin. The soiled bedding post-test, however, yields more visually pronounced differences between sterile water and zinc gluconate mice, and the zinc gluconate mice typically sniff each bin less and return to each bin less frequently than the mice treated with sterile water.

This is the behavior that I would expect if the zinc gluconate injections successfully induced anosmia in this group (Bester-Meredith, 2017; Duncan-Lewis et al., 2011; Slotnick et al., 2007).

Maternal Recognition

According to the graph of total behaviors, maternal recognition of an animal's own pup was higher in mice treated with sterile water than with zinc gluconate (Figure 8). Mice treated with sterile water spend a higher percentage of their time with their own pups than with foreign pups. This preference likely means that the mice treated with sterile water recognized their own pups, as has been shown in other species (Beach et al., 1956; Pillay, 2000). Furthermore, their recognition was maintained across time, as evidenced by the consistent results through short-term and long-term tests. The zinc gluconate group also demonstrated consistency across time, although they did not appear to prefer their own pups over foreign pups. While this measurement gives a general idea of the subject's preference, total behavior does still include both active and inactive behaviors. Looking at specific behaviors, and even total active behavior, will likely add nuance to this initial relationship.

With touching, the mice treated with sterile water appear to prefer their own pups more than the zinc gluconate mice do (Figure 9). Furthermore, in the days after birth, this preference seems more pronounced than it did for total behaviors. This effect decreases, if not disappears, in the days and weeks after the pups are weaned. In fact, on post-wean day 2, the mice treated with sterile water spent an average of less than 50% of the total touching time with their own pup. However, touching is the only behavior for which this is the case: the other behaviors, including both the total behavior and total active behavior measurements, show the difference in preference between the two groups staying consistent or growing more pronounced over time. With digging, for example, the mice treated with sterile water appear to prefer their own pups throughout the

testing period, and consistently spent a greater proportion of their digging time by their own pup (Figure 10). However, for this specific behavior, the average proportions for zinc gluconate mice do not reach 50%, which at least partly accounts for that difference between the groups.

The moment in the touching data in which the zinc gluconate mice prefer their own pup more than the mice treated with sterile water do, is replicated on day 6 after birth for climbing behavior (Figure 11). On post-birth day 2 and post-birth day 10, the mice treated with sterile water spend a greater proportion of their total climbing time on the side of their own pup than the zinc gluconate mice. However, on post-birth day 6, zinc gluconate mice demonstrate a stronger preference for climbing on the side of their own pups than the mice treated with sterile water. There is no obvious reason why this could be the case on that particular day, other than the low sample size.

The trends apparent in the graph of total behaviors (both active an inactive) are just as present, if not more so, in the graph of active behaviors, which combined touching, digging, and climbing (Figure 12). Here, the difference between percent of time with own pup for the sterile water and zinc gluconate groups averaged between 10.1 and 24.1%. This means that the mice treated with sterile water demonstrated a preference for their own pup that was between 10 and 24% greater than that of zinc gluconate mice. There was a concern that because the absolute total of behaviors included inactive behavior in which the mouse was on the side of the pup without being near the mesh barrier, or even facing it, that those totals may not indicate maternal recognition as much as active behaviors alone. However, a measure of total behaviors is still useful. There is a certain degree to which simply being on the own pup's side could constitute preference, modeled after mate fidelity experiments (Gubernick & Nordby, 1993; Gubernick et

al., 1994). This study may add credence to that idea as well, given that the trends are similar for total behavior and total active behavior.

One puzzling thing about these data is that the preferences exhibited in the short-term tests appear to carry over into the long-term. This was unexpected, because in previous studies on other rodent species such as the African striped mouse, there appears to be a time limit on maternal recognition (Pillay, 2000). However, African striped mice are not in the *Peromyscus* genus, and while they do share female aggression, they are not monogamous and biparental (Schradin, 2006). One reason for the consistency in this study may be that the subject mice were becoming more familiar with their own pups post-weaning, because they were exposed to one another during the maternal recognition tests. However, the post-weaning tests were spaced far enough apart that this should not have occurred, and the subject mice were not exposed to any pups between tests. There were some instances in which the same *own* pup had to be used repeatedly, as well as the same foreign pup, because of a lack of other pups of the same age. In this case, if the subject became too familiar with its own pup within the maternal recognition test environment, it should also have become familiar to a similar degree with the foreign pup. In that way, the relationship with the two pups should have struck a balance, accounting for potential bias.

Ecologically, retention of maternal recognition may be a byproduct of retention of mate preference. While Gubernick and Nordby investigated sexual fidelity by female California mice, they tested the females four hours after parturition, and each test was conducted only once (Gubernick & Nordby, 1993). However, prior to testing, the mated pairs had all raised at least one litter, which implies that the test results would have been consistent throughout their long-term relationship. Their results stated that in terms of quiet contact, females preferred their own

mates (Gubernick & Nordby, 1993). While the results of our maternal recognition experiment could not be analyzed for statistical significance, the measured behaviors were modeled off proximity and quiet contact, and were carried out over the first two months of a litter's life. Based on the similarities of these two experiments' methods and results, their underlying mechanisms could be related as well.

To further explore the field of parental recognition and parental memory, there are a variety of adjustments to be made in the future. Regarding whether these results are sex-specific, as California mice are biparental, I would expect to see similar trends for males and females. While this experiment's maternal recognition cages were modeled after those used to investigate mate fidelity (Gleason et al., 2012), making the test a more explicit choice could involve the use of a Y-maze (Gubernick & Nordby, 1993). If a more arena-like cage were to be used, as in this experiment, it may be useful while analyzing the maternal recognition videos to note behaviors that suggest a high degree of aggression, such as excessive biting. Measuring ultrasonic vocalizations may also be useful, if the mice exhibit different sounds when communicating with kin versus attacking an intruder. One could even remove the dividers between subject and pups altogether, if there were a way to allow the mothers physical contact while preventing infanticide. This would undoubtedly allow for more precise results, as one could measure actual performance of maternal behavior, rather than indicators of it.

Based on the trends shown in the olfaction and maternal recognition data, anosmic mothers prefer their own pups less than mothers retaining their sense of smell do. This trend is consistent across the different behaviors measured, and across time. The difference in preference between mothers who can and cannot smell is present shortly after birth, continues through the first month of the pups' lives, and is still evident through the month after they are weaned. While

these results are not statistically significant, their consistency resembles that suggested by mate preference studies. The mechanisms behind these two behaviors may be related as well, and exploring the nuances of maternal recognition may provide further insight into general social recognition.

Table 1

Ethogram describing behaviors measured in the olfaction tests (apple and bedding) and the maternal recognition tests.

Test	**Behavior**	**Description of Behavior**
Maternal Recognition		
Active	Touching	The mouse's nose points toward the mesh of either side and its entire body (minus its tail) is in the third of the section closest to the mesh, and/or the mouse's nose is immediately under or by the mesh, and/or the mouse is biting the mesh
	Digging	The mouse digs by the mesh with its paws or nose
	Climbing	The mouse climbs on the mesh such that its front two paws, or all four paws, are off the ground
Inactive	Other	The mouse's entire body (minus its tail) is in the third of the section closest to the mesh, but its nose is not pointing toward the mesh, nor is it digging or climbing
	Not visible	Due to the camera angle, the mouse's specific behavior is not discernible
Olfaction		
Apple test	Finding the apple	The mouse picks up the apple, bites the apple, or uncovers the apple by sniffing or digging.
Bedding test	Returns	After the mouse's first visit to any bedding bin, the mouse returns at any time to perform any behavior directed toward the bin
	Sniffing	The mouse sniffs the contents of the bin, usually with head down and movement of whiskers
	Digging	The mouse digs at the bedding in the bins with both front paws or head
	Sitting	The mouse directly sits in one of the bins, for more than 1 second, intentionally (running over the top of the bin or using the bin as leverage does not qualify)
	Dumping	The mouse purposefully hits or tips the bin, which may remove some or all of the bin's contents
	Moving	The mouse actively moves the bin from its original location with head or paws

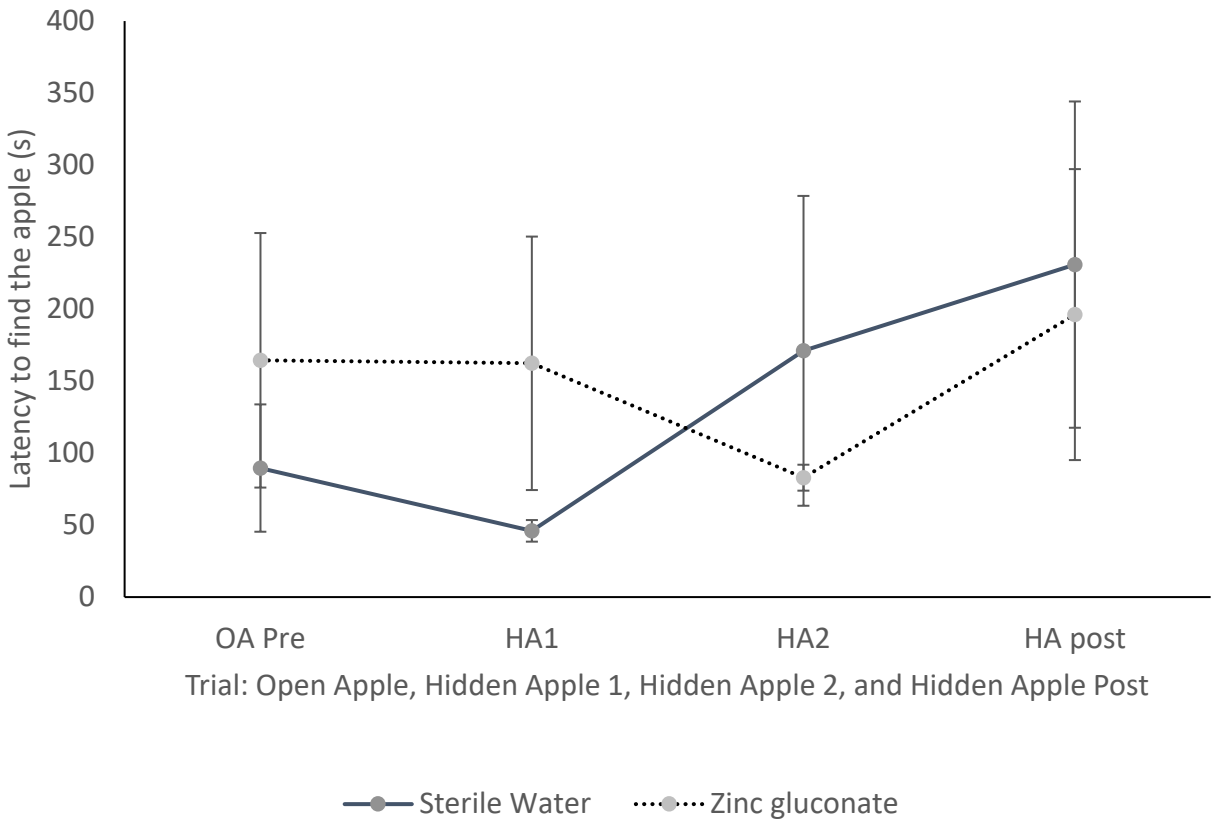

Figure 1. The mean latency to find the apple in all four apple tests: open apple pre-test, hidden apple pre-test 1, hidden apple post-test 1, and hidden apple post-test. Error bars represent standard error.

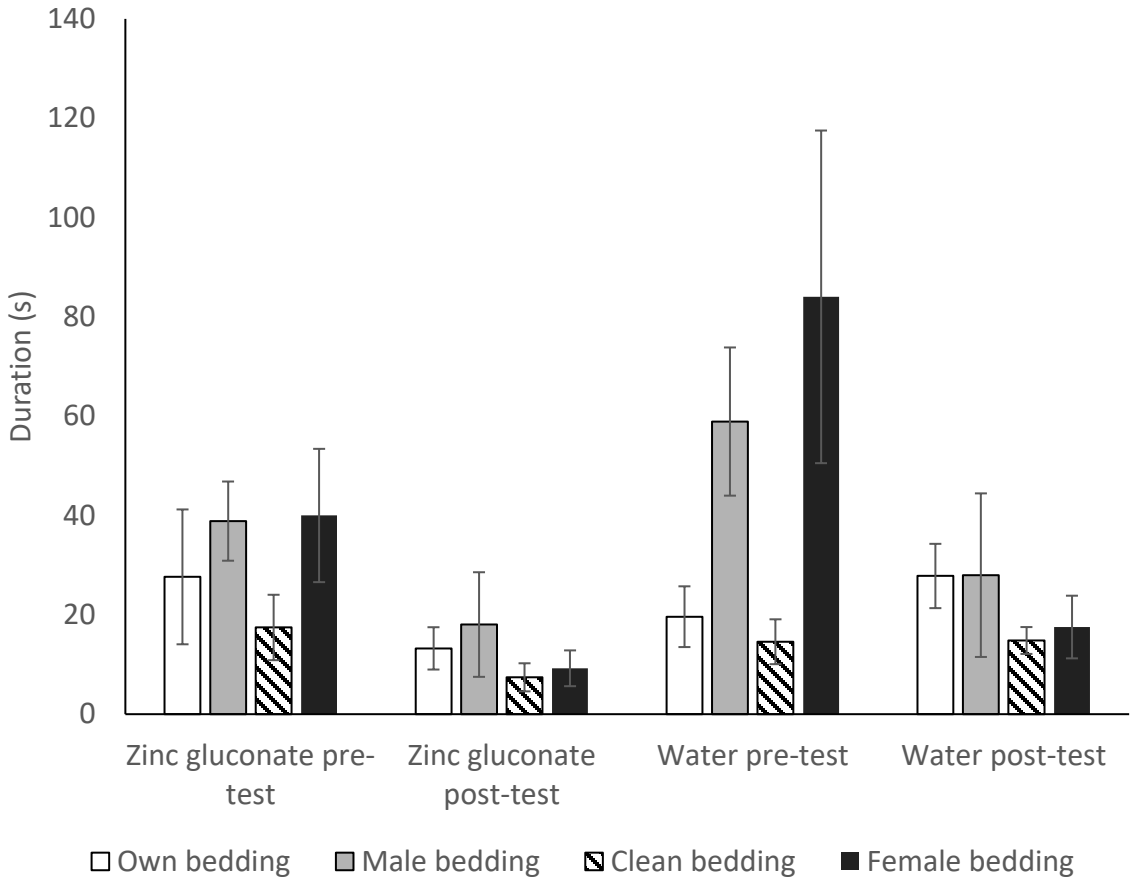

Figure 2. The mean duration measurements for sniffing between the soiled bedding pre-test and soiled bedding post-test. Error bars represent standard error.

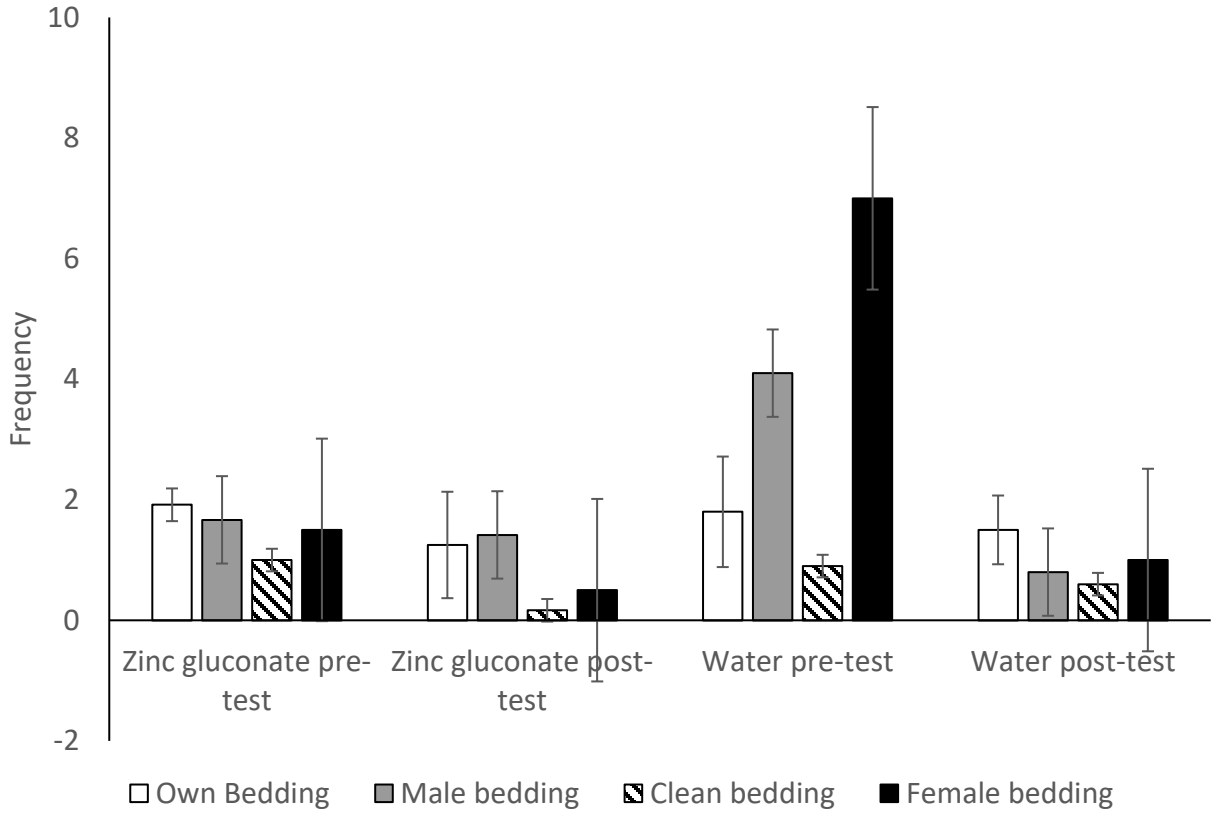

Figure 3. The mean frequency measurements for digging between the soiled bedding pre-test and soiled bedding post-test. Error bars represent standard error.

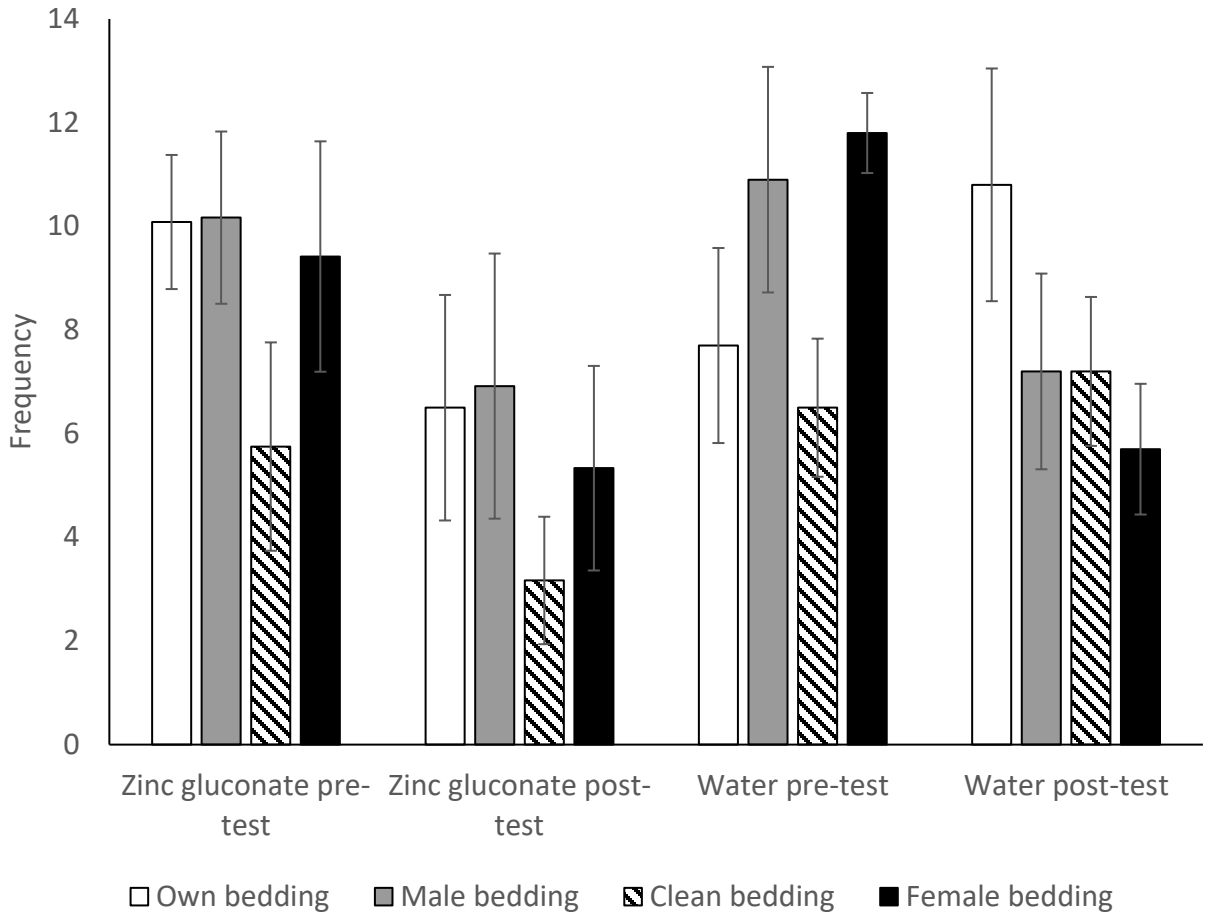

Figure 4. The mean frequency measurements for returns between the soiled bedding pre-test and soiled bedding post-test. Error bars represent standard error.

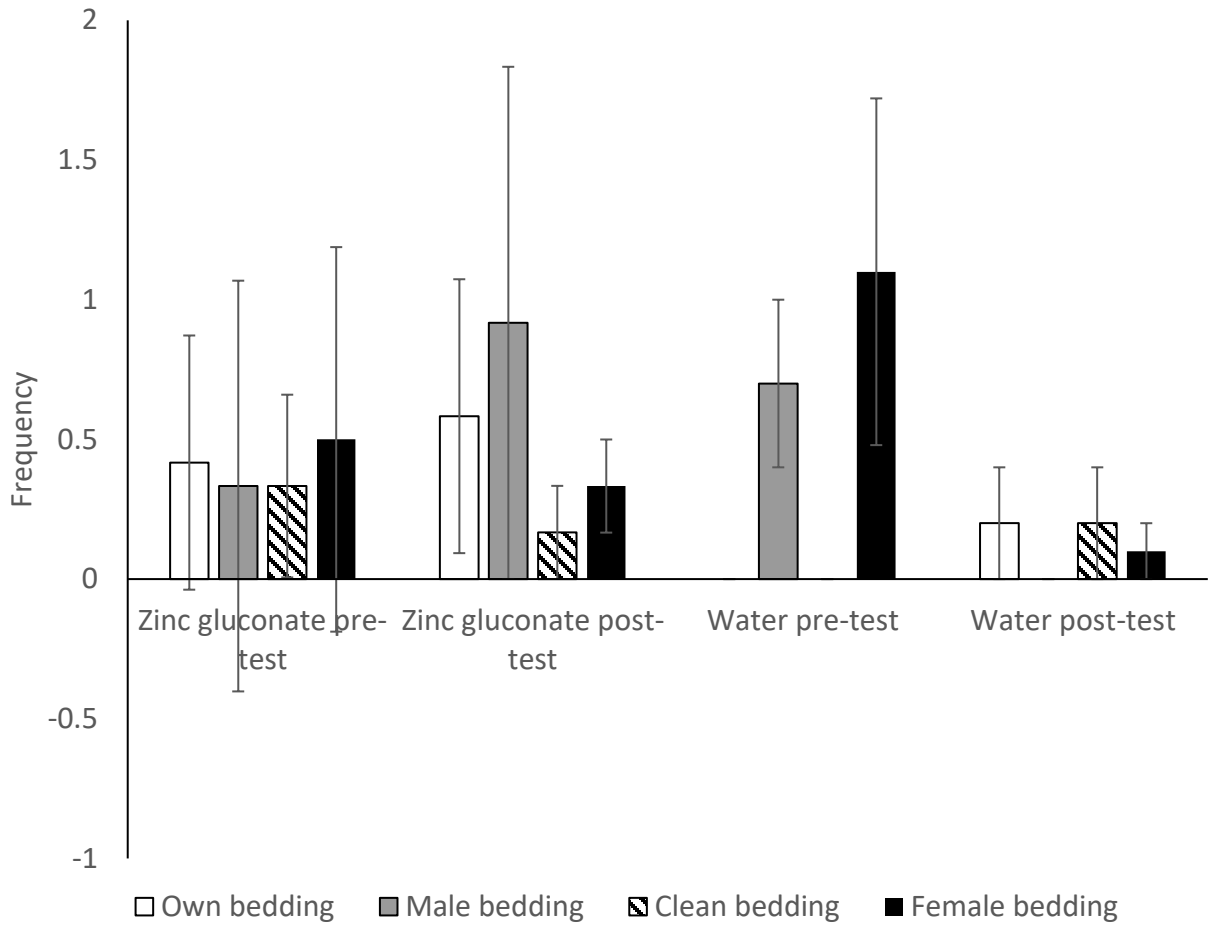

Figure 5. The mean frequency measurements for sitting between the soiled bedding pre-test and soiled bedding post-test. Error bars represent standard error.

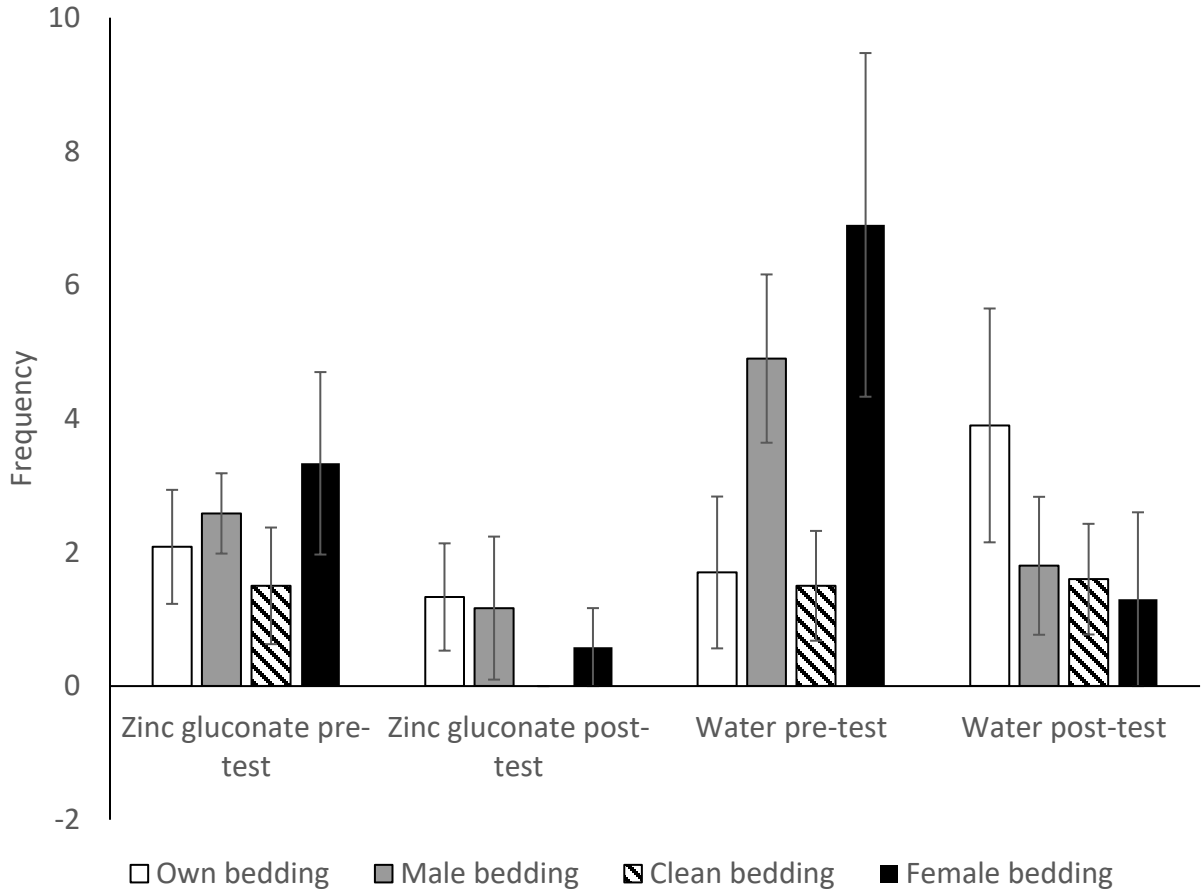

Figure 6. The mean frequency measurements for dumping between the soiled bedding pre-test and soiled bedding post-test. Error bars represent standard error.

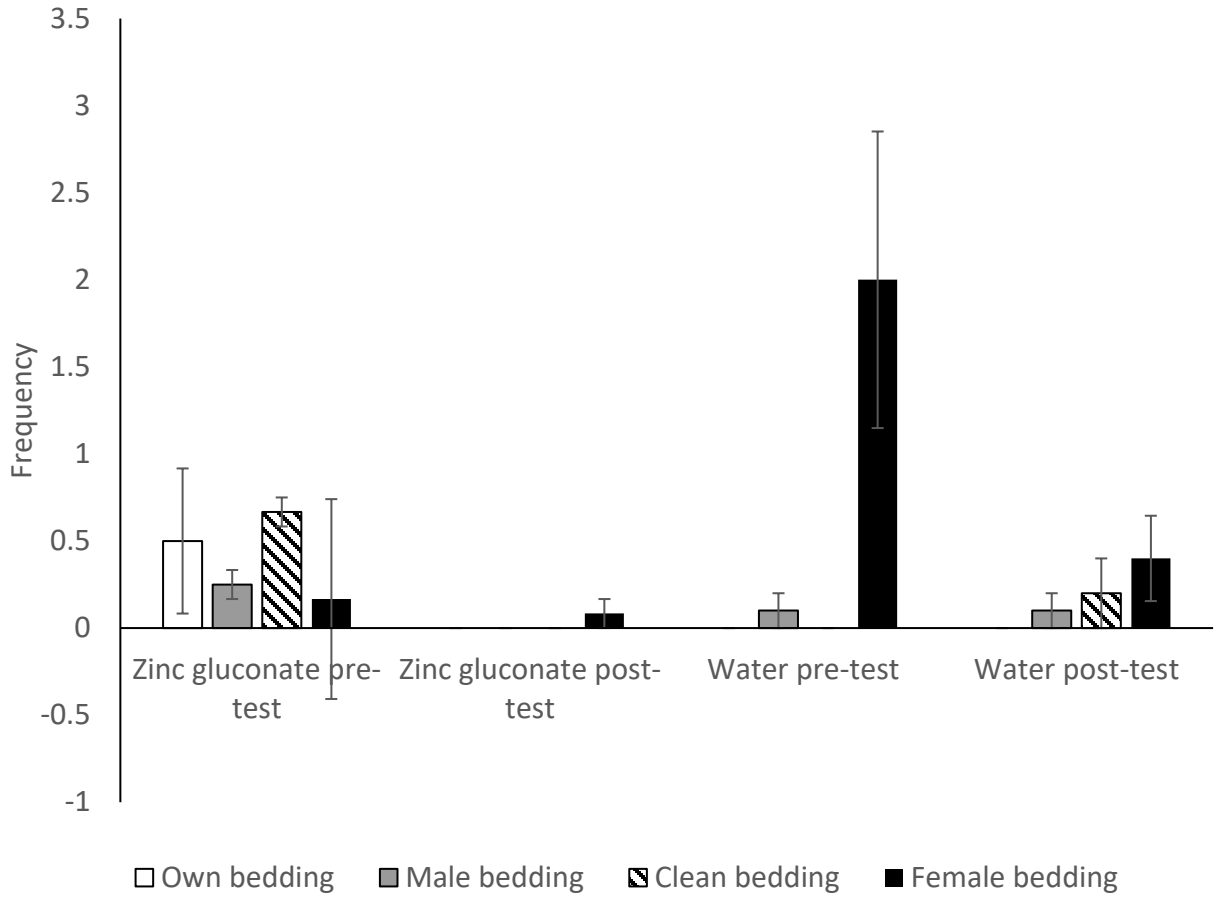

Figure 7. The mean frequency measurements for moving object between the soiled bedding pre-test and soiled bedding post-test. Error bars represent standard error.

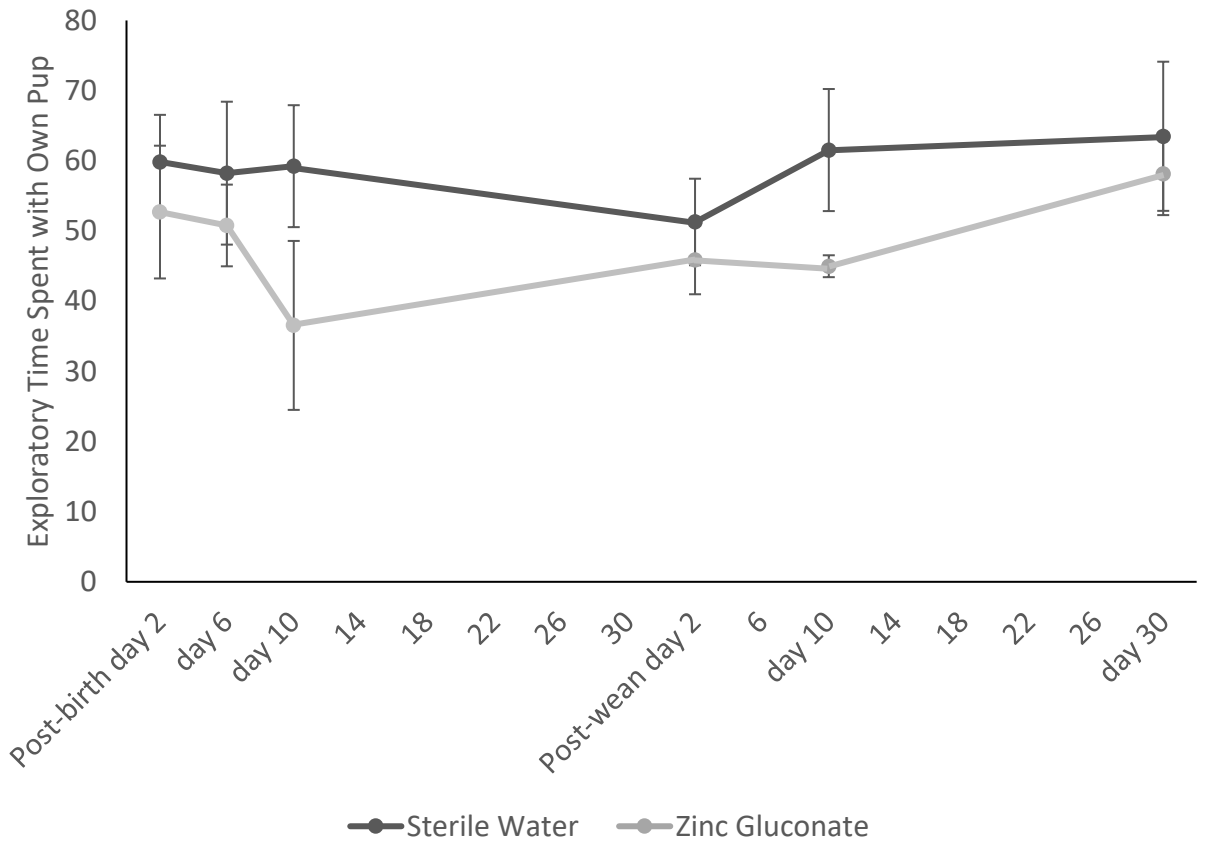

Figure 8. The mean percent of exploratory time spent with own pup for zinc gluconate and sterile water groups: all behaviors (active and inactive). Error bars represent standard error.

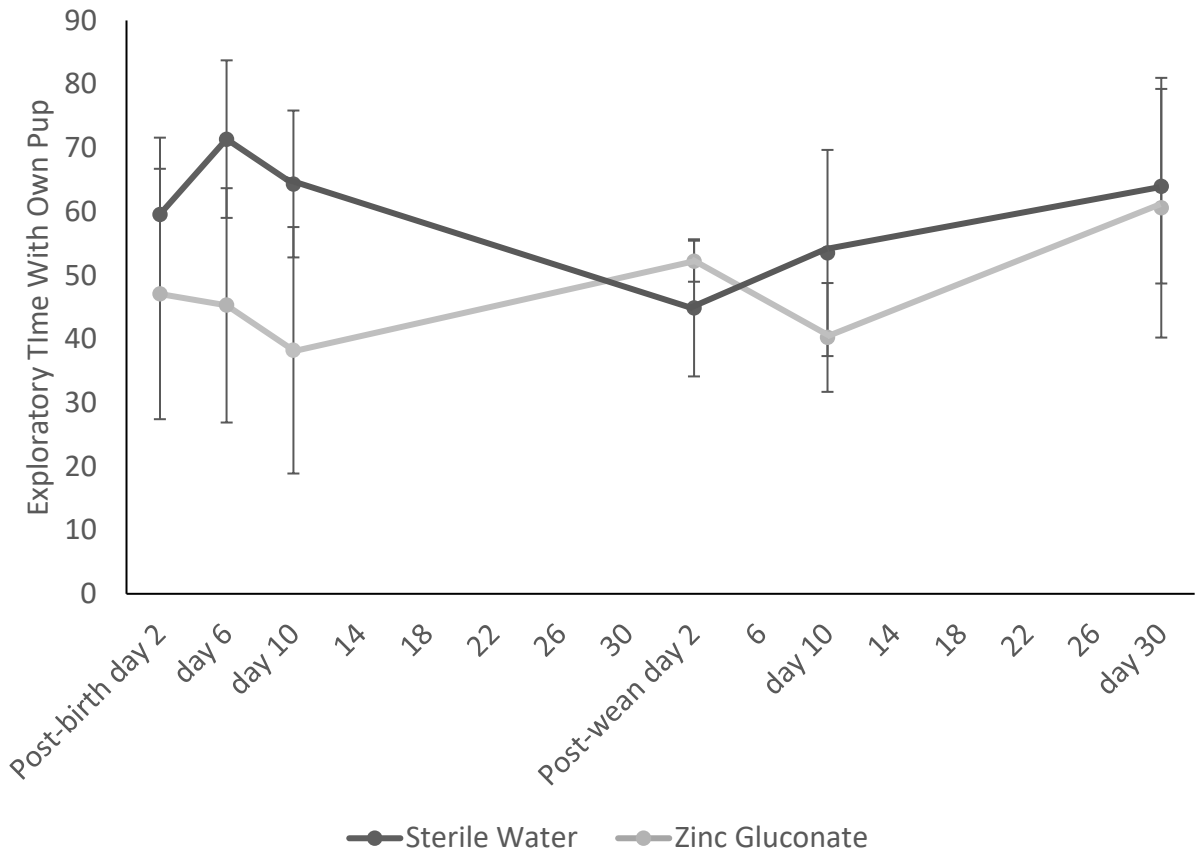

Figure 9. The mean percent of exploratory time spent with own pup for zinc gluconate and sterile water groups: touching. Error bars represent standard error.

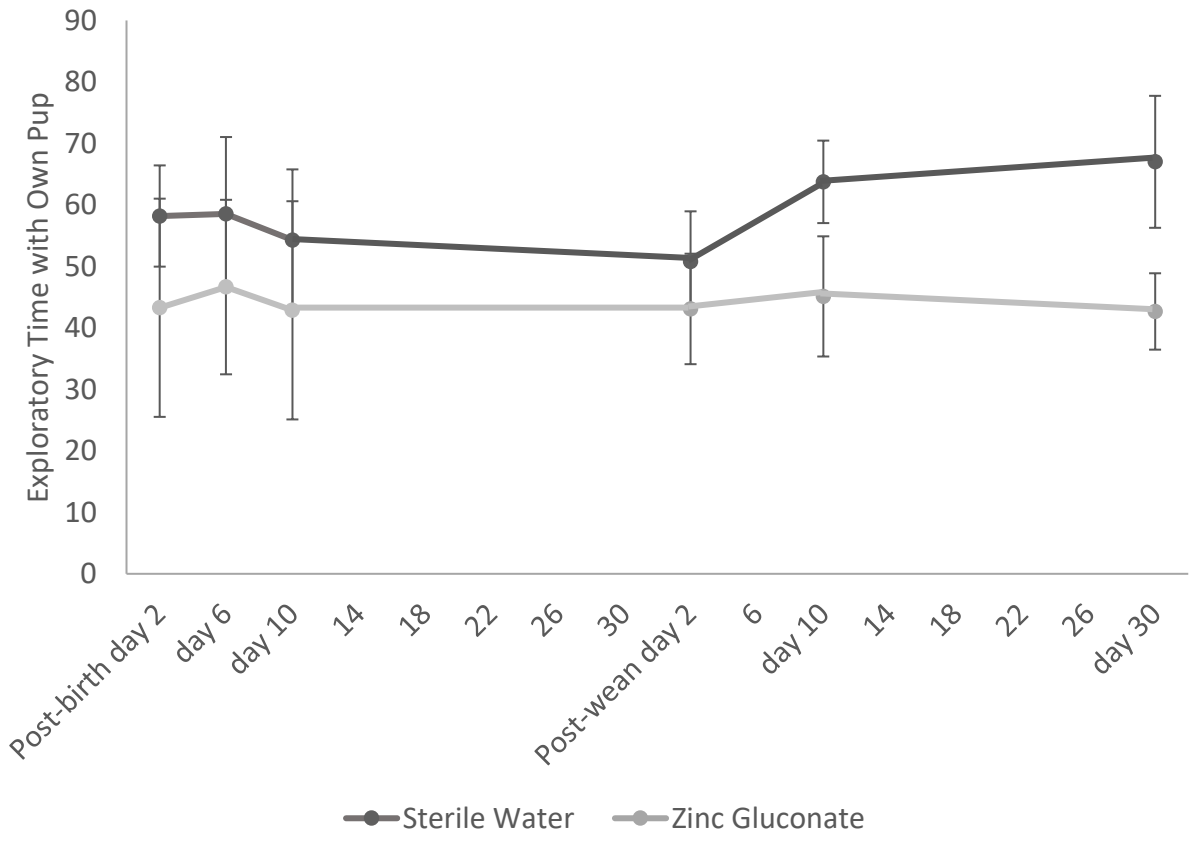

Figure 10. The mean percent of exploratory time spent with own pup for zinc gluconate and sterile water groups: digging. Error bars represent standard error.

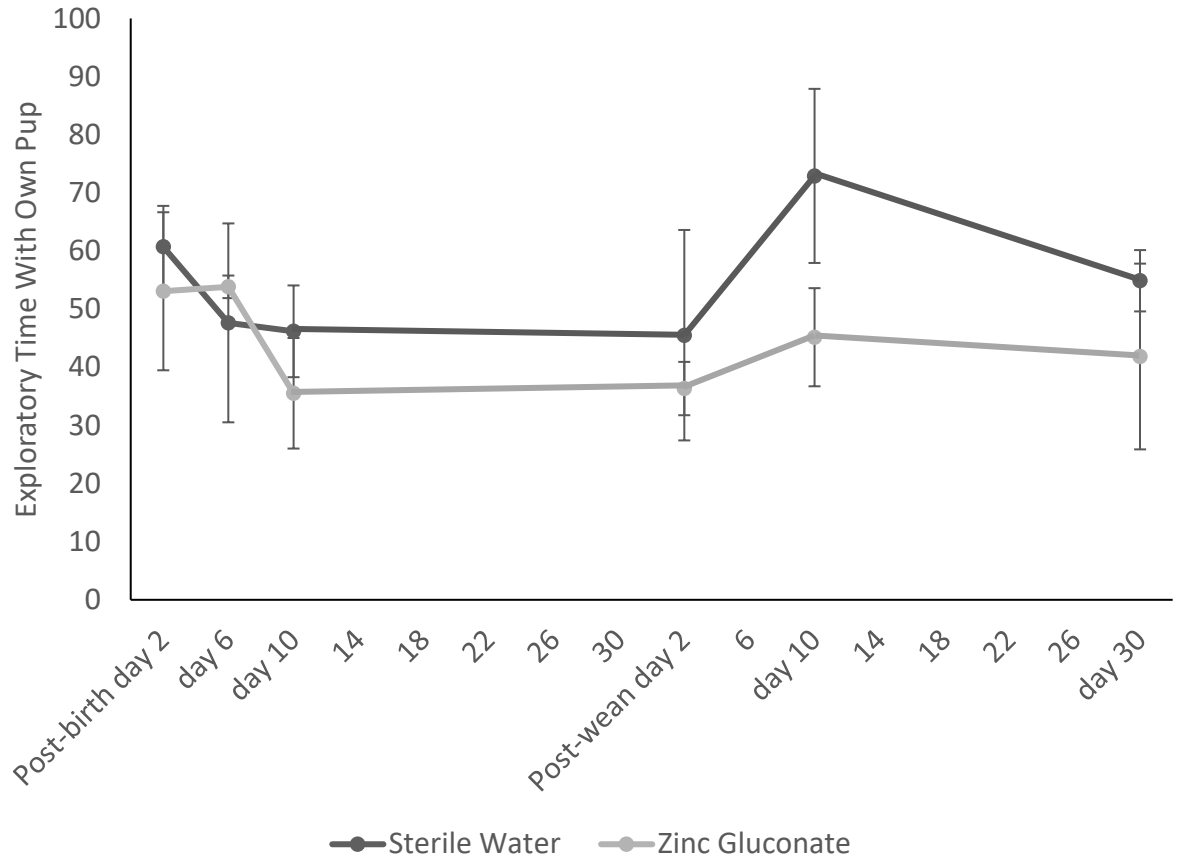

Figure 11. The mean percent of exploratory time spent with own pup for zinc gluconate and sterile water groups: climbing. Error bars represent standard error.

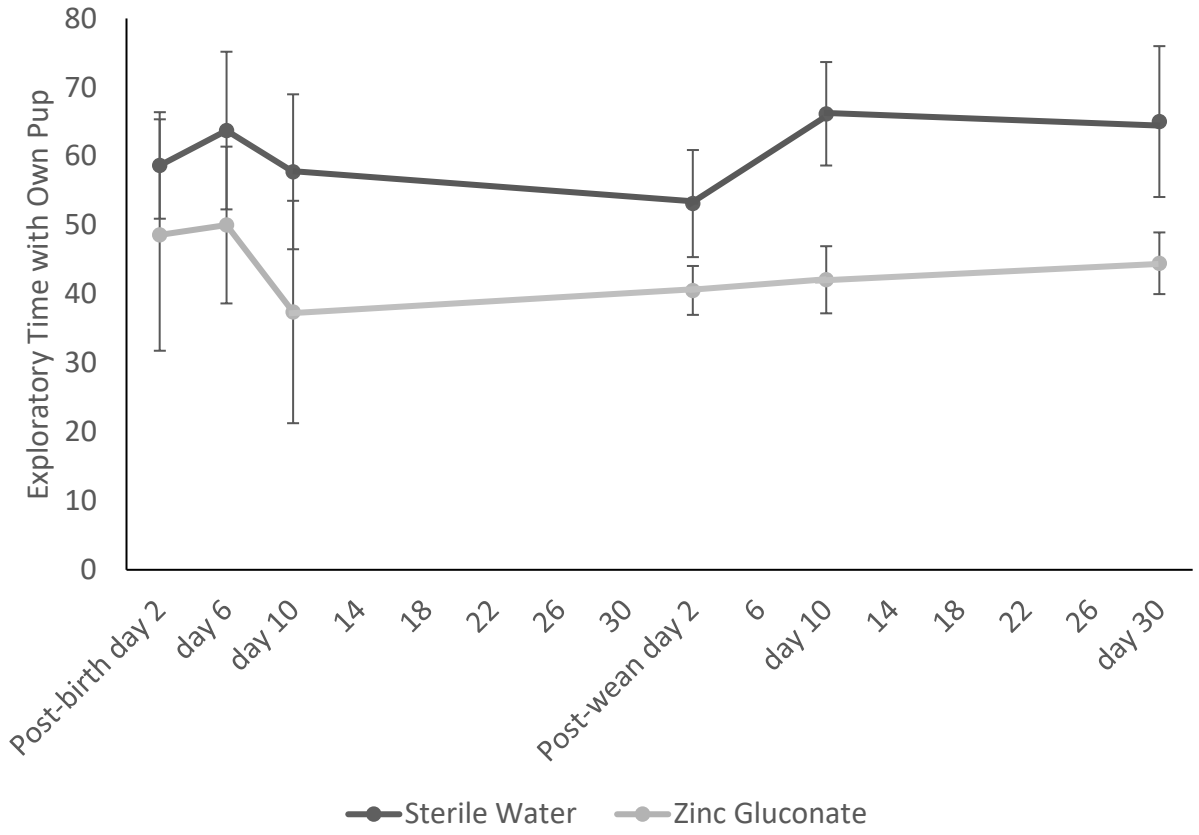

Figure 12. The mean percent of exploratory time spent with own pup for zinc gluconate and sterile water groups: all active behaviors. Error bars represent standard error.

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