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Placentophagia in the California Mouse (*Peromyscus californicus*): Causes and Consequences

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy in

Evolution, Ecology and Organismal Biology

by

Juan Pablo Perea-Rodriguez

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Dissertation Committee:

Dr. Wendy Saltzman, Chairperson
Dr. Theodore Garland, Jr.
Dr. Mark A. Chappell
Dr. Brian C. Trainor

The Dissertation of Juan Pablo Perea-Rodriguez is approved:

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_____

Committee Chairperson

University of California, Riverside

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Chapter 1: **Differences in placentophagia in relation to reproductive status in the California mouse (*Peromyscus californicus*)**
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A mi hermosa Familia

I dedicate my work to the ones I love.

ABSTRACT OF THE DISSERTATION

Placentophagia in the California Mouse (*Peromyscus californicus*): Causes and Consequences

by

Juan Pablo Perea-Rodriguez

Doctor of Philosophy, Graduate Program in Evolution, Ecology, and Organismal Biology
University of California, Riverside, June 2016
Prof. Wendy Saltzman, Chairperson

Male parental care, in contrast to maternal care, is uncommon in mammals, with an estimated 10% of species showing some level of male involvement in the raising of their young. Because males do not gestate or lactate, it is noteworthy that some of the same endocrine mechanisms underlying maternal care have been linked to the onset and maintenance of paternal care. In some biparental mammals, such as the monogamous California mouse (*Peromyscus californicus*), fathers, in addition to mothers, ingest placenta during the birth of their young. Still unknown, however, are the factors that activate placentophagia in males, as well as its functional consequences, if any. Especially intriguing is the possibility that placentophagia by males might modulate parental responsiveness, as reported in females. Thus, my dissertation investigated the possible facilitating role of male placentophagia in the expression of paternal behaviors, as well as the effects of mate-related stimuli on attraction to newborns and/or placenta.

The first experiment investigated possible influences of reproductive condition on placentophagia by determining the prevalence of placenta ingestion by males that were virgins, first-time expectant fathers, or experienced fathers. The second and third experiments were designed to determine possible neural, affective, and/or behavioral changes in male mice induced by oral administration of placenta. Finally, the fourth experiment investigated possible effects of chemical signals in excreta of gestating females on males' attraction to pups and placenta. I hypothesized that placentophagia would be more prevalent in parents, that placenta administration would lead to physiological, emotional, and behavioral changes that positively correlated with a males' parental responsiveness, and that exposure to excreta from a gestating female would increase the prevalence of placentophagia by virgin males.

These studies revealed that male California mice are more likely to ingest placenta when their mates become pregnant and/or with reproductive experience; however, these effects do not appear to be mediated exclusively by chemical cues from female excreta. They further indicate that placentophagia by males leads to decreased neophobia and/or anxiety. Together, these studies suggest that placentophagia may be an important component of males' transition to fatherhood in biparental mammals.

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DISSERTATION INTRODUCTION

Parental care is present in all mammalian species, with mothers bearing most of the responsibility. In some species (e.g., Norway rats [*Rattus norvegicus*], sheep [*Orvis spp*]), females transition from an averse response towards offspring-related stimuli to attraction, in association with mating, pregnancy, parturition, and/or lactation, suggesting that the neural circuitry processing infant-related stimuli, or the motivation to interact with such stimuli, changes when females reproduce (Numan & Insel, 2003). Not surprisingly, the onset of maternal behaviors has been linked to the endocrine, neural, and affective changes related to mammalian reproduction (Brunton & Russell, 2008; Numan & Insel, 2003). Specifically, the central and peripheral endocrine changes that occur during the different stages of mammalian reproduction facilitate the expression of maternal care by priming females to respond positively to offspring-related stimuli, ultimately allowing females to behave maternally towards their young (Numan & Insel 2003).

These hormonal changes involve several steroids (i.e., estradiol [E2], progesterone [P4], and glucocorticoids [GC]) and peptide hormones (prolactin [PRL], arginine vasopressin [AVP], and oxytocin [OT]) known to fluctuate during mating/pairbonding, pregnancy, and the post-partum period (Brunton & Russell, 2008; Numan & Insel, 2003). Furthermore, mothers experience changes in peripheral/central hormone and/or hormone-receptor availability in specific regions of the limbic system, a neural circuit important for social behavior. The bed nucleus of the stria terminalis (BST), medial preoptic area (MPOA), paraventricular nucleus of the hypothalamus (PVN), and amygdala have been implicated in the modulation of maternal motivation

through changes in hormone signaling. It has been proposed that the neuroendocrine changes induced by pregnancy change how these brain substrates respond to offspring-related stimuli (Brunton & Russell, 2008). For example, rat mothers have higher numbers of estrogen receptors (ER) in the ventral nucleus of the BST, the MPOA, and the medial amygdala (MeA), compared to non-mothers (Numan & Insel 2003). Lesion experiments have shown that the MPOA is essential for the expression of maternal care, whereas the PVN, BST, or amygdala play a supportive, but secondary role (Numan & Insel, 2003).

An aspect of mammalian reproduction that has been shown to enhance maternal responsiveness is the process of ingesting the afterbirth during/after parturition (i.e., placentophagia). Placentophagia is common among mammals, with approximately 73% of species showing maternal placentophagia, excluding cetaceans, pinnipeds, and humans, among others (Arendt et al., in review). The adaptive significance of placentophagia is has not been identified, but several hypotheses have been postulated. The most common explanations proposed suggest females (and their offspring) benefit from ingesting placenta because it reduces predation and/or pathogen transmission, helps mothers regain homeostasis, and/or results in reduced pain sensitivity (i.e., hypoalgesia) (Kristal, 1991). This hypoalgesic effect is mediated by changes in opioid signaling and can occur as soon as 5 minutes after ingestion (Doer & Kristal, 1989). The mechanism appears to be highly conserved, as oral administration of human and dolphin placenta to female rats also results in the above-described hypoalgesic effects (Kristal, 1991). Another interesting possibility is that placentophagia is a behavior that allows for the resolution of any conflict between parents and offspring in how much resources are

allocated to current, versus future reproductive effort (i.e., resolution of parent-offspring conflict [Trivers, 1974]) (Arendt et al., in review). In this scenario offspring are the main benefiters of their parental placentophagia because it increases the amount of care parents are willing to invest in their current reproductive effort, positively influencing offspring survival. In concurrence with this, a recent comparative study on the prevalence of placentophagia in over 140 species of mammals revealed that maternal placentophagia is more common in species that have lower reproductive effort (i.e., litter size x mass of a single newborn), suggesting that placentophagia is more common in species where high amounts of pre- and post- partum care/investment are required to reproduce (Arendt et al., in review).

The behavioral and physiological changes that result from ingesting placenta and/or amniotic fluid have been explained by the interactions between the complex hormonal content in the afterbirth and the nervous system. Such interactions are thought to prepare mothers to respond maternally towards their newborns. The placenta is an endocrine organ important for fetal nutrition and development, and it is derived in all cases of placental mammals from extra-embryonic tissues (trophoblast), with the addition of maternal uterine tissues in some taxa. This variation in embryonic and maternal layers that make up placentas is referred to placenta invasiveness, and it is directly related to the amount of connectivity between fetal and maternal circulation systems (Johnson & Everitt, 2000). In some cases placentas deeply invade maternal tissues during implantation (e.g., all primates except lemurs and lorises, dogs, cats, rabbits, mice), whereas in other cases (e.g., pigs, sheep, cows) the conceptus remains in a "free living"

stage where it is not attached to the uterus initially, but will implant much later, compared to species where invasive implantation occurs (Johnson & Everitt, 2000). Thus, placentas can be categorized by the number of placental layers originating from the trophoblast, as well as by their overall shape. In one extreme, humans produce haemomonochorial placentas, which only have a single trophoblastic layer, whereas dogs, bears and cats have placentas with three layers originating from the trophoblast (i.e., haemotrichorial) (Johnson & Everitt, 2000). These differences in the number of embryonic and maternal layers can have interesting implications in the resolution of parent-offspring conflict during the gestational period, as placentas with more fetal membranes are thought to have more "control" over how much resources are allocated to offspring by mothers (Haig, 1993). Regarding the shape of placentas, for species with invasive implantation, they are categorized by a round, disc-shaped placentas, aptly named *discoid* placentas. Most carnivores, on the other hand, produce *zonary* placentas, which are shaped like a belt surrounding the conceptus. In the case for non-invasive implantation placentas are separated into *cotyledonary* and *diffuse* placentas; these placentas are characterized by having multiple small sites of attachment to the uterine tissue. Since implantation does not occur in these species until later the conceptus is nourished by "uterine milk" until implantation occurs later on (Johnson & Everitt, 2000).

Placentas have specialized cells that produce a variety of biologically active chemicals that promote implantation, and the growth and development of the fetus, as well as help prepare mothers and fetuses for parturition. In order for survival of the conceptus to happen implantation has to occur. This is dependent on the recognition of

the conceptus by the mother, which is done mainly by secretion of chorionic/placental gonadotropins synthesized by the blastocyst, specifically by the syncytiotrophoblast (Johnson & Everitt, 2000). As mentioned previously, the mammalian gestation period is generally characterized by changes in several steroid and protein hormones. Of importance to the development of the fetus, as well as for the expression of proper maternal behaviors, are changes related to P4, E2, OT, and PRL. Changes in these hormones reflect important events essential for the survival of offspring. For some primate and rodent species, including humans and mice, peripheral levels of P4 are maintained high throughout most of the gestation period, initially by the corpus luteum and ultimately by the placenta (Johnson & Everitt, 2000). P4 is an important growth hormone that allows the embryo to develop. During this period, first-time gestating females normally don't show any maternal responsiveness mainly by the inhibitory effects of P4 (Numan & Insel, 2003). During most of the gestation period E2 remains low, but increases in availability toward the very end of the gestational period, at which time P4 secretion ceases (Numan & Insel, 2003). It is this change in E2 and P4 availability that has been shown to positively affect maternal care in rodents (Numan & Insel, 2003). As mentioned above, E2 secretion normally promotes interactions between females and newborns (Numan & Insel, 2003). Of importance to the development of the fetus is the essential role the placenta has in protecting it from the high levels of stress hormones (i.e., glucocorticoids). In humans, cortisol secretion increases towards the end of gestation, and birthing itself is a stressful event. Placentas produce an enzyme that inactivates glucocorticoids, Parturition is initiated by increased secretion of OT by

placental tissues and by the birthing process itself (i.e., vaginocervical stimulation). OT promotes contractions, which are essential for the expulsion of the neonate through the birth canal (Johnson & Everitt, 2000). The expulsion of the neonate is a very important event that promotes bonding between mothers and their offspring, as increased levels of OT have been heavily linked to social attachment in primates and rodents (Numan & Insel, 2003).

In some species (i.e., sheep, rabbits [*Oryctolagus cuniculus*]), maternal placentophagia is essential for mother-offspring bonding (Levy et al., 1983, Melo & González-Mariscal, 2003). As described above, the placenta is an endocrine organ that produces many peptide and steroid hormones, many of which have been shown to regulate maternal care (e.g., P4, E2, GCs, PRL, OT: Johnson & Everitt, 2000); thus, it has been proposed that the hormonal content of ingested placenta further primes females to respond positively to their young (Kristal, 1980, 1981). In rats, new mothers allowed to eat placenta had elevated circulating prolactin levels 1 day post-partum and decreased progesterone levels 6-8 days post-partum compared to new mothers that were not allowed to ingest placenta (Blank & Friesen, 1980). These hormonal changes are thought to increase maternal behavior and positively affect lactation. Additionally, orogastric administration of amniotic fluid to virgin female rats enhanced the facilitating effect of opiates on the maternal sensitization process (Neumann, et al., 2009). Furthermore, placentophagia and maternal care seem to be regulated by the same neural substrates, as electrical lesions of the MPOA abolish maternal behaviors and placentophagia in mothers, but not in virgin females (Noonan & Kristal, 1979). Although placentophagia by

humans is uncommon (Young & Benyshek, 2010, but see Coyle et al., 2015. Marraccini & Gorman, 2015), a handful of qualitative studies on the possible consequences of placentophagia exist. These studies have several methodological problems (e.g., no control groups, based on self-reports), which limits the interpretation of the results. Nevertheless, these studies suggest that human mothers may benefit from ingesting placenta (Hammett, 1918, Hammett & McNeile, 1917a, 1917b, Soyková-Pachnerová et al., 1954).

Paternal care, or any investment by males (potential fathers) in their (biological or perceived) offspring aside from sperm, is quite rare among mammals. In approximately 10% of species, mainly rodents, primates and carnivores, males, in addition to females, play an important role in raising their young (i.e., biparental care) (Kleiman & Malcom, 1981). Based on work on biparental birds, Lack (1968) suggested that natural selection would select for paternal care whenever two individuals (i.e., a monogamous pair) will raise more offspring together (resulting in higher Darwinian fitness) than they would by themselves. Other hypotheses for the presence of paternal care relate to reduced maternal aggression towards their offspring (Maestripieri & Alleva, 1991), differences in investment due to the mode of fertilization (Trivers, 1974), and male territoriality (Trivers, 1974), among others.

Lactation is very costly for female mammals (Hanwell & Peaker, 1977), and pup-directed care by males can reduce the overall energetic burden of reproduction in females (e.g., Walton & Wynne-Edwards, 1997), which can ultimately lead to shorter inter-birth intervals (Cantoni & Brown, 1997, Ribble, 1991) and increased pup survival (Dudley,

1974). Additionally, the presence of fathers can have direct effects on their offspring's behavior. Studies in voles show that the absence of fathers can influence their offspring's cognition, emotions, and reproductive behaviors. For prairies voles the presence of fathers significantly enhanced their performance in the Barnes maze (used for spatial learning and memory) (Ahern & Young, 2009). Female prairie voles that were raised by their only mother had deficiencies in alloparenting (Ahern & Young, 2009), pair-bonding, and parental behaviors (Ahern & Young, 2009), compared to female offspring that had been raised by both parents. Male offspring raised without a father also displayed deficits in pair-bonding (Ahern & Young, 2009). Similarly, in mandarin voles (*Lasiopodomys mandarinus*) social recognition in a habituation-dishabituation paradigm was impaired in both sexes of paternally deprived offspring (Cao et al., 2014).

Male mammals, as stated earlier, do not experience the hormonal changes females undergo throughout gestation, parturition, and lactation, so the mechanisms behind the onset and maintenance of paternal behaviors may be different than in females. A likely scenario is that similar neural and endocrine pathways are used by both sexes (Reburn & Wynne-Edwards, 1999), but the way these pathways are activated to yield parental behaviors may be different (Wynne-Edwards & Timonin, 2007). For this reason, much of the research on the physiological mechanisms of paternal care has investigated the roles of hormones involved in maternal care, which has produced inconsistent findings in a number of biparental species (Saltzman & Zeigler, 2014, Wynne-Edwards & Timonin, 2007). As mentioned earlier, lesion studies on biparental California miceshow the essential role the MPOA in regulating male caretaking behaviors (Lee & Brown 2002,

2007), as well as the medial amygdala, in the case of prairie voles (Kirkpatrick et al., 1994), and similar to what is found for the case of maternal care, the BST, PVN, and the basolateral and central amygdala play a secondary, although important role in mediating paternal care.

Much attention has been given to the potential role of testosterone (T) and other androgens in paternal care. Commonly, T has been implicated in agonistic and sexual behaviors (Simpson, 2001). In many cases, males (especially those that breed seasonally) may have to concurrently express paternal behaviors towards their offspring, sexual behaviors towards their mates, and aggressive behaviors towards intruders or rival males in order to breed successfully. Thus, one of the main questions relating to paternal behavior is, what is the mechanism by which males with high T can also behave paternally towards their offspring One hypothesis involves aromatization of T to E2 (Trainor et al., 2003), which results in activation of ER (or different ER subtypes [Genazzani et al., 2006]), as well as the direct effect of T through androgen receptors. These distinct signaling pathways (T and E2) may therefore mediate a variety of behaviors under similar hormonal conditions. Testosterone in some male rodents (and birds; Beletsky et al., 1992, Wingfield et al., 1990), commonly promotes mating behaviors when converted to E2 by aromatase (Trainor & Marler, 2002).

Studies have shown a negative relationship between T and paternal care in several species (e.g., prairie vole, *Microtus ochrogaster*: Wang & De Vries, 1993; black-tufted-ear marmoset, *Callithrix kuhlii*: Nunes et al., 2001). In contrast, experimental studies have yielded contradictory findings and suggest a positive relationship between T and

paternal behaviors in the Djungarian hamster (*Phodopus campbelli*: Schum & Wynne-Edwards, 2005), the volcano mouse (*Neotomodon alstoni*) (Luis et al., 2009), and the California mouse (Trainor & Marler, 2002). These data suggest that species-specific differences in how T affects how males respond to pup-related stimuli exist.

Other likely candidates that may influence the onset of paternal care are E2 and P4. Human first-time fathers show higher levels of plasma E2 when compared to controls (Berg & Wynne-Edwards, 2002). Rosenblatt and colleagues successfully induced paternal behaviors in castrated male rats after priming them with E2 and P4 and injecting them with E2 1 day later (Rosenblatt et al., 1996), or by bilateral implants of E2 (Rosenblatt & Ceus, 1998). These data suggest that E2 may play a role in the activation of paternal behaviors under similar hormonal background to what a gestating female would experience. In contrast, in the biparental Djungarian hamster, castration failed to inhibit paternal behaviors in primiparous males (Hume & Wynne-Edwards, 2005). Additionally, for male Djungarian hamsters, serum E2 levels do not appear to fluctuate with reproductive condition (Schum & Wynne-Edwards, 2005), and ER alpha-immunoreactivity in several brain regions (BST, MPOA & medial amygdala), does not appear to differ between fathers and non-fathers (Timonin et al., 2008). In contrast, mandarin vole (*M. mandarinus*) fathers have lower levels of ER alpha-immunoreactivity in the medial preoptic area and the BST, and higher levels in the ventromedial hypothalamus, compared to non-fathers (Song et al., 2010). These results do not rule out the possibility that effects may result from E2 produced in the brain via aromatization of circulating T (Trainor et al., 2003), but osmotic infusion of aromatase inhibitor in the

prairie vole had no effect on paternal behavior, suggesting that in this species paternal care is not E2-dependent (Hume & Wynne-Edwards, 2006). In contrast, the activation of paternal behaviors in California mouse fathers is dependent on E2, potentially resulting from the aromatization of T in the MPOA (Trainor & Marler, 2002, Trainor et al., 2003). Research on the potential role of P4 in the onset and maintenance of paternal behaviors is scarce and contradictory. In the uniparental house mouse (*Mus spp.*), P4-receptor knockouts show increased pup-directed care and decreased aggressive behaviors towards pups by mated males (Schneider et al., 2003). Additionally, mice treated with the progesterone receptor antagonist RU486, showed reduced aggression toward pups and enhanced paternal behaviors, whereas progesterone treatment of wild-type males significantly increased aggression toward pups (Schneider et al., 2003).

In contrast, a study in which the authors "mimicked" P4 withdrawal experienced by late-pregnant females in virgin male mice (as experienced by females in late-pregnancy) showed that exposure to P4 followed by P4 withdrawal had no significant effect on paternal behaviors but increased infanticidal behaviors towards pups in non-paternal behaving males (Schneider et al., 2009). Consistent with this, although correlational, California mouse fathers have lower circulating levels of P4 when compared to male mice cohabitating with a pregnant female, or with a lactating female and pups, and virgin males (Trainor et al., 2003). In contrast, Djungarian hamsters show an increase in P4 levels the day prior to birth of their litter, which lasts until one day postpartum (Schum & Wynne-Edwards, 2005). Further experiments are needed to determine the direct role that P4 may play in the onset of paternal behaviors.

A clear understanding of the relationship between stress and paternal care is still lacking. Correlational data show that both human and Djungarian hamster fathers experience decreased baseline levels of GCs before birth of their offspring (Berg & Wynne-Edwards, 2002, Reburn & Wynne-Edwards, 1999). These findings might suggest that a reduction in GC levels might allow parents to increase investment in their offspring and decrease the likelihood of litter abandonment. Data on oldfield mice (*Peromyscus polionotus*) and Wied's marmosets (*Callithrix kuhlii*) support this hypothesis, showing decreased offspring survival or paternal care with high paternal GC levels (Good et al., 2005, Nunes et al., 2001). On the other hand, acute elevation of corticosterone had no effect on paternal behaviors in California mouse fathers (Harris et al., 2011). As GCs start to decline during the early post-partum period no difference in GC levels is found between breeding and non-breeding male California mice; similar findings are also seen in male prairie voles (Campbell et al., 2009, Chauke et al., 2011).

As mentioned above, several peptide hormones/neuropeptides have been implicated in maternal care, and these may function similarly in males, with some important exceptions (see below). In rodents and primates, PRL has been shown to have a positive relationship with paternal behaviors. Male Mongolian gerbils show increased peripheral PRL levels when paired, and these levels increase steadily when pups are present (Brown et al., 1995). In a similar manner, California mouse first-time fathers show higher levels of PRL when compared to males whose mates are pregnant with their first litter and virgin males (Gubernick & Nelson, 1989). Djungarian hamster expectant fathers show a similar increase one-day prepartum (Reburn & Wynne-Edwards, 1999). In

humans paternal PRL levels have been positively related with play behavior towards infants (Gordon et al., 2010). Similarly, male meerkats (*Suricata suricata*) that provide paternal or alloparental care show increased plasma PRL levels before babysitting (Carlson et al., 2006). In contrast, experimental studies in the biparental Djungarian hamster, in which PRL was manipulated by the use of a PRL inhibitor (dopamine agonist), do not support the positive relationship between peripheral PRL and paternal behaviors (Brooks et al., 2005). A methodological problem that arises with manipulation of PRL is that this hormone is normally inhibited by dopamine, a neurotransmitter heavily involved in the reward system and voluntary movement.

Other peptide hormones such as AVP and OT are likely candidates as mediators of paternal care, since they are involved in a wide array of social behaviors. As discussed earlier, both AVP (and increased expression of its V1a receptor: Lim et al., 2004) and OT facilitate (or positively correlate with) maternal behaviors (Bosch & Neumann, 2008) and have been implicated in facilitating pair-bonding behaviors as well (Cushing & Carter, 1999). Insel & Young (2000) proposed that OT is mostly involved in the onset of maternal care and AVP in paternal care. This is supported by studies in California mice, in which no difference in central or peripheral OT in relation with fatherhood was detected (de Jong et al., 2009, Gubernick et al., 1995). Direct injection in the lateral septum of an AVP-V1a receptor antagonist decreased paternal behaviors in virgin male prairie voles (Wang et al., 1994). Interestingly, AVP increases paternal responsiveness in the meadow vole (*M. pennsylvanicus*), non-monogamous, faculatively biparental species (Parker & Lee, 2001). These data suggest that AVP or its receptors may be upregulated in

fathers, but this increase in AVP receptor expression can also be a result of behaving paternally (Young et al., 1999). Importantly, peripheral AVP is most commonly involved in osmoregulation. Males and females will normally ingest their pups' urine and feces, which may result in osmotic stress, therefore the increase in AVP can be a response to this osmotic challenge (Wang et al., 1994). Additionally, due to the similarities in their structure OT and AVP may interact with each other's receptors (Carter, 1998).

Another potentially important mechanism that has been proposed to facilitate the onset of male parental care is the ingestion of placenta during/after parturition. Placentophagia by fathers has been described in several biparental species (dwarf hamster: Jones & Wynne-Edwards, 2000; California mouse: Lee & Brown, 2002a; prairie vole: K.L Bales, pers. comm.; common marmoset, *Callithrix jacchus*, cotton-top tamarin, *Saguinus oedipus*: T. E. Ziegler, pers. comm.; silvery marmoset, *C. argentata*: J. A. French, pers. comm.; and some human populations: Coyle et al., 2015), but the physiological and behavioral consequences are unknown. Additionally, the mechanism that promotes the onset of placentophagia (and paternal behaviors) in male California mice is still unknown. As stated above, male mammals do not undergo the same endogenous (neuro)endocrine changes as females at the onset of parenthood, so placentophagia may have important implications for the initiation of paternal behavior (Gregg & Wynne-Edwards, 2005, 2006). Furthermore, the onset of paternal behaviors and placentophagia may arise through the same mechanisms since they are proposed to be expressed during the mate's late pregnancy or early Postpartum period, and may be mediated by similar neural substrates (Kristal, 1980).

I addressed the possible role that placentophagia may have in facilitating male parental care in the California mouse, addressing three questions: 1) what are the possible stimuli or events that promote placentohagia?, 2) what (if any) are the consequences of male placentophagia?, and 3) are the effects of placentophagia dependent on reproductive condition? These questions address possible functions of male placentophagia in this species, and will seek to identify the possible mechanism by which these changes may occur. I hypothesized that male placentophagia results in direct physiological and behavioral changes that promote caretaking behavior in new fathers, and that exposure to chemical signals from a geststing female increases attraction to placenta in males. The specific aims of the dissertation are as follows:

Aim 1: Does reproductive condition of adult males and females affect their propensity to engage in placentophagia?

Aim 2: Does ingesting placenta lead to behavioral and/or physiological changes in males?

Aim 3: Does placentophagia modify pain sensitivity and/or exploratory behavior in males?

Aim 4: Do chemical signals from gestating females modulate placentophagia and/or caretaking behavior in males?

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Chapter 1

Differences in Placentophagia in Relation to Reproductive Status in the California Mouse (*Peromyscus californicus*)

Juan P. Perea-Rodriguez and Wendy Saltzman

Department of Biology, University of California, Riverside, CA 92521, USA

Abstract

Parturient females ingest placenta in most mammalian species, whereas fathers may do so in species in which both parents provide care for their offspring. To determine if the propensity to eat placenta varies with reproductive status in the biparental California mouse, we presented placenta to virgin (housed with a same-sex pairmate), expectant (pregnant with their first litter), and multiparous adult males and females. Liver was presented identically, 3-7 days later, as a control. Multiparous females were more likely to eat placenta than expectant and virgin females (p-values <0.016), whereas both multiparous and expectant males had higher incidences of placentophagia than virgins (p-values <0.016). Liver consumption did not differ among groups within either sex. These results suggest that propensity to eat placenta increases with maternal/birthing experience in females, and with paternal experience and/or cohabitation with a pregnant female in males.

Keywords: California mice, biparental care, placentophagia, reproductive condition, paternal care

Introduction

Placentophagia, or the process of ingesting placenta (and amniotic fluid) during and after parturition, is common among mammals, with only a few exceptions (humans: Young & Benyshek, 2010; semi-aquatic and aquatic mammals, camelids: Young, et al., 2012). This behavior has been proposed to enhance maternal responsiveness, potentially by priming the mother's brain through the diverse hormonal content found in placenta (Kristal et al., 2012; Melo & González-Mariscal, 2003). Studies on rabbits (*Oryctolagus cuniculus* L.; González-Mariscal et al., 1998), rats (*Rattus norvegicus*; Kristal et al., 1981a), sheep (*Ovis aries*; Lévy & Poindron, 1987; Lévy et al., 1983), and dogs (*Canis lupus familiaris*; Abitbol & Inglis, 1997) have shown that the presence of amniotic fluid on newborns enhances mother-offspring bonding and advances the onset of maternal behaviors. In a similar manner, virgin female rats, which normally do not express maternal behavior spontaneously, show increased attraction to pups and decreased latency for maternal sensitization when presented with unrelated, placenta-smeared pups as compared to unrelated pups that were not treated with placenta (Kristal et al., 1981a).

Females can also show physiological changes after eating placenta that can ultimately affect their behavioral responses towards their offspring. Primiparous female rats allowed to eat placenta while giving birth show increased plasma prolactin concentrations one day postpartum, as well as decreased plasma progesterone concentrations 6-8 days postpartum, as compared to primiparous mothers that were not allowed to eat placenta (Blank & Friesen, 1980). These hormonal changes can potentially promote maternal care (e.g. lactation, licking/grooming of offspring; Numan & Insel,

2003), and have been proposed to facilitate the mother's return to regular estrous cycling (Blank & Friesen, 1980). Furthermore, placenta contains high levels of endogenous opioids and opioid-enhancing factors, which increase the pain threshold of mothers during parturition by enhancing opioid-mediated analgesia (Kristal et al., 1985), and, as a result, might reduce the time spent in labor. These studies indicate that placenta contains active substances that can alter the physiology and behavior of individuals that ingest it.

Interestingly, females respond differently when presented with placenta depending on their reproductive state. Female rats are mostly averse to placenta when sexually inexperienced, but become attracted to it during and after pregnancy (Kristal et al., 1981a). The highest incidence of placentophagia is seen towards the end of gestation (Kristal et al., 1981b), and in a similar fashion, female rats become placentophagous with induced pseudopregnancy (Steuer et al., 1987). Additionally, lesioning of the medial preoptic area, a brain region implicated in the onset and expression of parental behaviors (paternal behavior: de Jong et al., 2009, Lee & Brown, 2007; maternal behavior: Olazábal et al., 2001, Rosenblatt & Ceus, 1998) as well as other behaviors (partner preference: Kindon et al., 1996, Paredes et al., 1998; sexual behavior: Harding & McGinnins, 2004, Markowski et al., 1994, Powers et al., 1987), inhibits placentophagia in parturient rats (Noonan & Kristal, 1979). These results suggest that the physiological changes that females undergo during pregnancy promote the ingestion of placenta, amniotic fluid and attached membranes. Furthermore, these findings point to similarities in the neural processes and substrates that mediate the onset of parental behavior and placentophagia. Interestingly, placentophagia can also be facilitated by social cues (i.e., the presence of a

parturient or placentophagous female rat increases attraction to placenta in a female observer; Kristal & Nishita, 1981).

In a handful of biparental species (i.e., species in which both parents provide care for their offspring), males, in addition to females, readily ingest placenta during the female's parturition (dwarf hamster, *Phodopus campbelli*: Jones & Wynne-Edwards, 2000; California mouse, *Peromyscus californicus*: Lee & Brown, 2002; prairie vole, *Microtus ochrogaster*: K.L Bales, pers. comm.; common marmoset, *Callithrix jacchus*, and cotton-top tamarin, *Saguinus oedipus*: T. E. Ziegler, pers. comm.; silvery marmoset, *C. argentata*: J. A. French, pers. comm.). In the two species in which placentophagia by males has been best characterized, the dwarf hamster and the California mouse, fathers lick their parturient mate's anogenital region, clean and pull neonates as they are expelled, and ingest amniotic fluid and placenta in the process. In dwarf hamsters, the frequency of placentophagia does not change with age in sexually inexperienced males, but increases in expectant fathers on the day before their mate gives birth (Gregg & Wynne-Edwards, 2005). These changes in propensity to eat placenta by males mirror those seen in females; however, the mechanisms that enable males to become placentophagous are unknown. Increased incidence of placentophagia in reproductive males might be due to cohabitation with a female, mating, and/or exposure to changing chemical cues produced by females throughout their pregnancy (Jemiolo et al., 1994).

In this study we investigated the factors influencing the propensity for placentophagia in male and female California mice. This species is socially and genetically monogamous, and both males and females invest heavily in their offspring

(Gubernick & Alberts, 1989, Ribble, 1991). Data on the frequency of placentophagia throughout an individual's life history are lacking. Such data may be important for elucidating the factors influencing the expression of placentophagia as well as the potential role of placentophagia in the onset of parental behavior in this species (Jones & Wynne-Edwards, 2000). In the present study, therefore, we aimed to characterize the frequency of placentophagia in male and female California mice in different reproductive conditions. Specifically, we aimed to determine how social housing condition (same-sex groups vs. heterosexual pairs), parental experience, and pregnancy may affect an individual's propensity to eat placenta.

Each mouse was presented with freshly extracted, full-term placenta from an unrelated female on a single occasion. To determine if mice show changes in their attraction to placenta specifically or to highly vascularized tissues in general, we also presented animals with liver in a similar manner (Gregg & Wynne-Edwards, 2005, 2006, Melo & González-Mariscal, 2003). Consistent with the hypothesis that placentophagia facilitates the onset of parental behavior in both sexes (Gregg & Wynne-Edwards, 2005, 2006, Jones &Wynne-Edwards, 2000), we predicted that both males and females would show increased likelihood of ingesting placenta as they become sexually experienced, and would show further increases in placentophagia with pregnancy and/or parental experience. Additionally, we predicted that the prevalence of liver ingestion would not differ between the sexes or among reproductive conditions; we argue that although California mice mostly eat seeds (Merritt, 1974, Meserve, 1976), they are potentially likely to eat meat if the opportunity arises (pers. obs.).

Methods

Animals

California mice are medium-sized rodents (40-70 grams) found throughout most of coastal California, from San Francisco to the Baja Peninsula (Gubernick & Alberts, 2004). As mentioned above, they are genetically monogamous (Ribble, 1991) and breed throughout the year in the lab and in the wild, with gestation periods ranging from 29 to 34 days (average gestation length is 31.6 days [Gubernick, 1988]). California mice produce small litters containing 1-4 pups (average litter size: 2; Gubernick & Alberts, 2004). Their life expectancy in the wild is 9-18 months (Gubernick & Alberts, 2004) and they can live up to 2-4 years in captivity (C.A. Marler, pers. comm.; unpub. data). In our lab, their maximum recorded reproductive lifespan is 16-18 months (unpub. data).

We used mice that were born in our colony at the University of California, Riverside (UCR) and descended from animals purchased from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia, SC, USA). To minimize inbreeding, we avoid pairing males and females that are more closely related than second cousins. Mice were weaned at 27-32 days of age, prior to the birth of siblings. At weaning, animals were ear-punched for identification and housed in same-sex groups consisting of four age-matched individuals (littermates and/or unrelated). Some animals remained in these groups throughout the experiment (see below); others were placed in male-female pairs when they were at least 90 days old.

Mice were maintained as described previously (Chauke et al., 2011, Harris et al., 2011). Briefly, mice were housed in 44 x 24 x 20 cm polycarbonate shoebox-type cages

with aspen shavings and cotton wool (~ 5 g), and were provided with Purina Rodent Chow 5001 (LabDiet®, Richmond, IN, USA) and water *ad libitum*. Animals were kept on a 14:10 light:dark cycle with lights on at 0500 h and lights off at 1900 h. Room temperature and humidity were maintained at approximately 18-26°C and 60-70%, respectively. All of the procedures used were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the UCR IACUC. UCR is fully accredited by AAALAC.

Reproductive Conditions

Animals from each sex were grouped into the following three reproductive conditions:

Virgins: Virgin males (V-Males, n=11) and females (V-Females, n=10) had no prior sexual experience and had never been housed with a pup (except their own littermates) prior to testing. These animals were housed in groups of four age-matched, same-sex mice per cage. Virgins were 110-412 days old (V-Males: 259.4 ± 124.7, V-Females: 147.2 ± 32.1, mean ± SE) at the time of placenta testing. Males in this group included two littermates, whereas females included two mice from each of two litters from different lineages.

Expectant Parents: Expectant males (E-Males, n=10) and females (E-Females, n=11) had been paired for at least 21 days at the time of the placenta test, and the female was pregnant with the pair's first litter (i.e., primiparous). Thus, these individuals were sexually experienced, had no parental experience at the time of testing, and had never been housed with a pup other than their own littermates. Expectant males and females were tested 2-28 days prepartum (9.5 ± 8.4 days; average gestation length for California

mice is 31.6 days [Gubernick, 1988]). Pregnancy was monitored on the basis of typical weight increases seen in pregnant females in our colony (see below) and confirmed by subsequent parturition. Expectant parents were tested with placenta when they were 125-305 days of age (E-Males: 202.2 ± 64.2, E-Females: 202.2 ± 61.2) and with liver 3-7 days after. Two of the females and none of the males in this group were littermates.

Multiparous Parents: Multiparous males (M-Males, n=13) and females (M-Females, n=10) had produced multiple litters (range: 3-13 litters; 7.6 ± 6.7) and thus were both sexually and parentally experienced. These animals had 1- to 10-day-old pups living with them at the time of placenta testing, and females were likely to be pregnant, as this species undergoes postpartum estrus and copulates on the day of parturition (Gubernick, 1988; pers. obs.). M-Males and M-Females were 179-632 days old (M-Males: 406.6 ± 117.9, M-Females: 362.3 ± 129.9) when tested with placenta. This group contained no same-sex littermates.

Each animal was tested under only a single reproductive condition. We determined pregnancies (or the lack thereof) by weighing females twice weekly and monitoring them for sustained weight gain after pair formation or after they gave birth to a previous litter. Data from each animal were inspected for a gradual and sustained increase in weight, as well as a rapid weight increase during the last week of pregnancy (unpub. data).

Within each sex, age at the time of testing differed significantly among mice in the three reproductive conditions (females, $\chi^2=8.72$, $p<0.0001$: Kruskal-Wallis test; males, $F=11.26$, $df=2$, $p=0.002$; one-way ANOVA). Appropriate post hoc pair-wise

comparisons showed that M-Females were significantly older than E-Females and V-Females (p-values <0.05), whereas M-Males were older than E-Males and V-Males (p-values <0.05). No differences in ages were found between expectant and virgin animals from either sex (p-values >0.05)

Behavioral Tests

Each mouse underwent a single placenta test, followed 3-7 days later by a liver test. Approximately 30 min before each test, the animal's cagemate(s) were removed, while the test animal remained in the home cage. At the outset of the test, placenta or liver (~0.2 g; see below) in a small, hexagonal, plastic weigh boat (2.5 cm diameter x 0.95 cm deep) was placed in one end of the cage. The animal was videotaped for 10 min or until it consumed all of the tissue, whichever came first. The weigh boat and any remaining tissue were then removed from the cage, the tissue was reweighed, and the animal's cagemate(s) were returned. Behavior was later scored from videotapes using the JWatcher event-recorder program (Blumstein & Daniel, 2007). Tests were conducted in the colony-housing room during lights-on, between 1100 and 1800 h. The animals were not food-deprived prior to or during behavioral testing. When two mice from the same cage were tested on the same day, both/all cagemates were reunited in the home cage for at least 30 minutes following one animal's test, before the next focal animal was isolated in the home cage prior to testing. No more than three cagemates were tested in a single day.

Animals were considered placentophagous if they ate all or some of the experimentally presented placenta during the test, as determined visually (see below).

The same criterion was used for liver tests. In many instances dehydration or contact with the bedding dramatically changed the weight of the experimentally presented tissues. As a result, the post-test tissue weights were not reliable and thus were not used when categorizing animals as placentophagous or not. The proportion of individuals that ate each tissue (liver or placenta) as well as the latency to approach the tissue was determined.

A total of 8 videotapes (2 from placenta tests, 6 from liver tests) were lost due to a camera malfunction. As a result, the final sample sizes used for quantitative behavioral analyses for placenta tests or liver tests (i.e., latency to approach tissue) ranged from 8 to 12.

Tissue Procurement

Placenta: Placentas were harvested from pregnant females from our breeding colony (417.7 ± 39.3 days old) that had given birth previously to 1-13 litters. The test animals and donors were no more closely related than second cousins. All extracted placentas were close to full term (30-33 days after the previous birth). Pregnancies were monitored by weighing the females twice per week as described above. Donors were euthanized by CO_2 inhalation, and uterine horns were extracted immediately. Each amniotic sac was dissected individually, and fetuses were euthanized with sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, Michigan, USA; ~0.2 mL, i.p.). We lightly dried individual placentas and the adhering membranes by pressing them briefly onto paper towel, and then placed the tissues in plastic weighing boats with 1.5 mL of saline. Placentas were subsequently blotted lightly on a paper towel, cut into ~0.2 g sections, and

transferred to a clean, dry weighing boat immediately before being used in a behavioral test, which commenced 10-30 minutes after harvesting of the tissues.

Liver: Livers were harvested from adult virgin females (135 ± 54.5 days old) that were no more closely related to the test animals than second cousins. Donors were euthanized by CO_2 inhalation, and their livers were extracted, divided into ~0.2 g sections, lightly blotted, placed in plastic weighing boats with 1.5 mL saline, transferred to dry weighing boats, and presented to the test animal following the same procedures used for placenta. Again, behavioral tests commenced within 10-30 minutes following harvesting of livers.

Statistical Analyses

Data were analyzed nonparametrically using R version 15.0 (Vienna, Austria). To characterize differences in the prevalence of placentophagia or liver consumption among reproductive states and between sexes, pairwise comparisons were made using Fisher's Exact-Boschloo tests; the alpha values of these pairwise comparisons were Bonferroni-corrected to 0.016 ($\leq \alpha/n$, where n = number of pairwise comparisons; 0.05/3=0.016). The remaining analyses were evaluated using a critical p-value of 0.05 (2-tailed).

One-way ANOVAs (for normally distributed data) or Kruskal-Wallis tests (for non-normally distributed data) and appropriate pairwise post hoc tests (Tukey HSD or Dunn tests, respectively) were used to compare latencies to approach tissues. McNemar tests were used to determine differences in the propensity for individual mice within each reproductive condition to eat liver or placenta. Mann-Whitney U tests were used to

determine if age or number of days prepartum (expectant females and males only) differed among animals within each sex that did and did not consume placenta.

Results

Females

The prevalence of placentophagia differed markedly among females in the three reproductive conditions (Figure 1.1). Multiparous females (M-Females, 8 of 10) had the highest incidence of placentophagia, followed by expectant females (E-Females, 5 of 11) and virgin females (V-Females, 2 of 10). M-Females were significantly more likely to eat placenta than were V-Females ($p=0.012$; Fisher's Exact-Boschloo test). No significant differences in the prevalence of placentophagia were found between M-Females and E-Females ($p=0.18$; Fisher's Exact-Boschloo test), or between E-Females and V-Females ($p=0.29$; Fisher's Exact-Boschloo test, Figure 1.1).

In contrast to placentophagia, females' propensity to eat liver showed no significant pairwise differences among the three reproductive groups when we employed a Bonferroni-adjusted critical p-value of 0.016 (M-Females vs. E-Females, $p=0.52$; M-Females vs. V-Females, $p=0.042$; E-Females vs. V-Females, $p=0.29$; Fisher's Exact-Boschloo tests; Figure 1.1). Further analyses revealed that within each of the three reproductive conditions, individual females were equally likely to eat liver and placenta (all p-values >0.05; McNemar tests).

As described above (see Methods), females in the three reproductive conditions differed significantly in age. Therefore, to determine whether age might influence the

propensity of female California mice to eat placenta, we performed a Mann-Whitney U test comparing ages of all placentophagous E-Females and V-Females with those of all non-placentophagous E-Females and V-Females; we excluded M-Females since they both were significantly older than the other groups and had maternal experience. This analysis revealed that age did not differ significantly between females that ate placenta (n=7; 193.0 ± 22.5 days old) and those that did not (n=14, 168.0 ± 4.6 days old; U(20)=27, p=0.10; Mann-Whitney U test). Similarly, the number of days prepartum did not differ between E-Females that did (n=5; 10.2 ± 3.3 days prepartum) and did not (n=6; 10.0 ± 4.9 days prepartum) eat placenta (U(11)=13.0, p=0.78; Mann-Whitney U test).

The latency to approach liver differed significantly among females in the three reproductive conditions (χ^2=11.0, df=2, p=0.012; Kruskal-Wallis test). Specifically, M-Females and E-Females approached the liver more quickly than V-Females (p-values<0.05; Dunn's tests)

Males

Similar to females, males in the three reproductive conditions differed significantly in their propensity to eat placenta. Both M-Males (11 of 13) and E-Males (7 of 10) were significantly more likely to ingest placenta than V-Males (2 of 11; M-Males vs. V-Males: p=0.002; E-Males vs. V-Males, p=0.002; Fisher's Exact-Boschloo tests; Figure 1.2). The incidence of placentophagia did not differ significantly between M-Males and E-Males (p=0.51; Fisher's Exact-Boschloo test).

In contrast to placentophagia, the incidence of liver ingestion did not differ reliably among males in the three reproductive groups when we utilized the Bonferroni

corrected p-value (p=0.016) (M-Males vs. E-Males, p=1.0; M-Males vs. V-Males, p=0.046, E-Males vs. V-Males, p=0.07; Fisher's Exact-Boschloo tests; Figure 1.2). Further analysis revealed that M-Males tended to have higher rates of placentophagia than liver ingestion, but this trend was not significant (p=0.06; McNemar test). Males in each of the remaining two conditions were equally likely to eat placenta and liver (E-Males, p=0.25; V-Males, p=1.0; McNemar tests). Furthermore, males' latencies to approach placenta and liver did not differ among the three reproductive conditions (placenta: χ^2=1.06, p =0.59; liver: χ^2=0.34, p=0.84; Kruskal-Wallis tests).

As with females, we compared age at the time of placenta tests between E-Males and V-Males that did and did not eat placenta; again, we excluded M-Males because they were both significantly older than E-Males and V-Males and parentally experienced. In contrast to females, placentophagous males (n=9; 224.0 ± 13.4 days old) were significantly younger than non-placentophagous males (n=12 232.9 ± 1.5 days old; U(20)=20.0, p=0.047; Mann-Whitney U test). The number of days prepartum did not differ between placentophagous (n=7; 8.0 ± 1.0 days prepartum) and non-placentophagous E-Males (n=3; 13.0 ± 4.3 days prepartum; U(9)=8.0, p=0.66; Mann-Whitney U test).

Comparisons between males and females from the same reproductive condition showed no differences between the sexes in the propensity to ingest either placenta or liver (all p-values >0.1; Fisher's Exact-Boschloo tests).

Discussion

In this study we sought to characterize the incidence of placentophagia in male and female California mice in three different reproductive conditions (multiparous parents, expectant first-time parents, and virgins), and to determine how parental and/or sexual experience might influence this behavior. Our results indicate that both males and females differ in their propensity to eat placenta depending on their reproductive condition. In contrast, the incidence of liver ingestion was not affected by an animal's reproductive condition, suggesting that effects of reproductive condition are specific to placentophagia and not to ingestion of any highly vascularized tissue. Additionally, we found similar patterns of placentophagia, and liver ingestion in males and females from the same reproductive condition.

Among females, virgins showed the lowest incidence (20%) of placentophagia, whereas multiparous females showed the highest, with 80% of experienced breeding females eating some or all of the presented placenta. These results suggest that female California mice increase their attraction to placenta as a result of parenting experience and/or parturition, including previous exposure to placenta. Moreover, the finding that the prevalence of placentophagia did not differ between expectant and virgin females suggests that neither sexual experience nor pregnancy increases the propensity to ingest placenta in female California mice. Age also did not appear to be an important determinant of placentophagia, as age did not differ reliably between expectant and virgin females that did and did not eat placenta. This finding differs from results in female

dwarf hamsters, in which rates of placentophagia decreased with age (Gregg & Wynne-Edwards, 2005).

Although, pregnancy did not appear to affect the incidence of placentophagia in females, expectant females and multiparous females had significantly lower latencies to approach liver than virgin females. This finding suggests that pregnancy and/or lactation may decrease neophobia and increase exploratory behavior in females, which could be motivated by an increased need for food (Bartness, 1997, Johnstone & Higuchi, 2001). Neophobia and exploratory behavior may be influenced by the neuroendocrine changes that females undergo during gestation, parturition and lactation, as demonstrated in rats and mice (*Mus spp.*) (Numan & Insel, 2003).

For male California mice, parental experience and their mate's pregnancy seemed to increase attraction to placenta, as both multiparous males and expectant males showed significantly higher rates of placentophagia (84% and 70%, respectively) when compared to virgin males (9%). It is unclear if these results reflect the effect of copulation, cohabitation with a female, or sensory cues specifically from a pregnant female. Interestingly, however, we have found that males housed with females that, for unknown reasons, failed to become pregnant showed low prevalence of placentophagia (unpub. data), suggesting that cohabitation with a female is not sufficient to induce placentophagia.

In contrast to females, latency to approach either placenta or liver did not differ among males in the three reproductive conditions, suggesting that neophobia did not differ among male reproductive conditions (see also Chauke et al., 2012) and did not

contribute to differences in placentophagia. Moreover, since males from the three reproductive conditions were equally likely to eat liver, the high incidence of placentophagia in multiparous males and expectant males did not result from an increased attraction to vascularized tissues in general. Although California mice mainly eat seeds (Merritt, 1974, Meserve, 1976), they will eat meat opportunistically (pers. obs.), and this may explain why males (and females) from different reproductive conditions did not differ in the tendency to ingest liver. Furthermore, lesions of the lateral hypothalamus, which negatively affect ingestive behaviors, do not affect placentophagia in parturient female rats, suggesting that placentophagia is regulated by different mechanisms from those that control hunger (Kristal, 1973). Additionally, in contrast to females, younger expectant and virgin males were more likely to ingest placenta than older expectant and virgin males. Importantly, however, 7 out of 9 placentophagous males from these two groups were expectants, which tended to be younger than virgin males; thus, reproductive condition might play a larger role than age in influencing placentophagia. In dwarf hamsters, placentophagia is not affected by age in sexually inexperienced males (Gregg & Wynne-Edwards, 2005).

Several caveats should be kept in mind when interpreting the results of this study. First, because all animals were tested first with placenta and then with liver 3-7 days later, it is possible that the response to liver was influence by previous exposure to placenta. Second, we cannot rule out the possibility that the age differences found among groups within each sex might have contributed to the differences among groups in the propensity to ingest placenta, as suggested by the results of the age comparison between

placentophagous and non-placentophagous males. Nonetheless, as described above, we believe that age made little, if any, contribution to the observed differences in placentophagia. Third, all animals were separated from their cagemates prior to behavioral testing, and this procedure might have differentially effects on behavior in the different groups. In particular, if a strong pair bond exists between opposite-sexed pairmates, it might be expected that even brief disruption of the pair bond could alter the animals' performance in behavioral tests. In a previous study, we compared putative anxiety-related behavior and neophobia between breeding, expectant, and virgin male California mice (Chauke et al., 2012). As in the present study, males were removed from their cagemates shortly before testing. Very few behavioral differences were found among males that were pair-housed with either a postpartum female (and pups), a pregnant female, or a male; however, numerous differences were found between these animals and singly housed virgin males. These findings suggest that short-term separation from a male or female pairmate may not differentially alter behavior in male California mice. Unfortunately, we do not have comparable data for females. Finally, virgin males and females in the present study were not housed in pairs but in groups of four animals, which might have affected their responses to the behavioral tests.

In summary, our results show that the pattern of placentophagia in female California mice is similar to that in female rats (Kristal et al., 1980) and rabbits (Melo & González-Mariscal, 2003) in that females tended to increase their attraction to placenta with maternal experience. In contrast to findings in rats (Kristal et al., 1981), rabbits (Melo & González-Mariscal, 2003), dwarf hamsters (Gregg & Wynne-Edwards, 2005)

and Djungarian hamsters (Gregg & Wynne-Edwards, 2006), however, our results indicate that pregnancy alone does not increase placentophagia in female California mice. Among males, the prevalence of placentophagia was higher in expectant first-time fathers and in experienced fathers than in virgins, similar to findings in the biparental dwarf hamster (Gregg & Wynne-Edwards, 2005). Importantly, we found that high levels of placentophagia in males emerge with pregnancy of their mate (and potentially with sexual experience) and persist for at least several days postpartum.

Placentophagia has been shown to facilitate the onset of maternal behavior in some female mammals (Kristal, 1980, 2009). Placenta contains a variety of hormones that can potentially affect neuroendocrine activity in individuals that consume it and, as a result, might alter their behavior towards neonates (Kristal, 1980, 2009, Kristal et al., 2012). Although the specific hormones and relative amounts present in placenta vary among species, placenta has been reported to contain progestagens, estrogens, oxytocin, lactogens, corticotropin-releasing hormone, and opioids (Petraglia et al., 1996), all of which have been shown to influence maternal behavior in several mammalian species (Numan & Insel, 2003). The presence of maternally derived estrogens in placenta is of particular relevance, as estradiol has been shown to activate paternal behavior in California mice (Trainor & Marler, 2002). Further investigation is needed into the potential physiological and behavioral consequences of placentophagia in males to determine its possible role in the onset of paternal behaviors.

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Figure 1.1: Proportion of female California mice that ingested placenta (black bars) and liver (white bars) among multiparous (M-F), expectant (E-F), and virgin females (V-F). Numbers within bars represent sample sizes. * - $p<0.016$ (Fisher's Exact-Boschloo test).

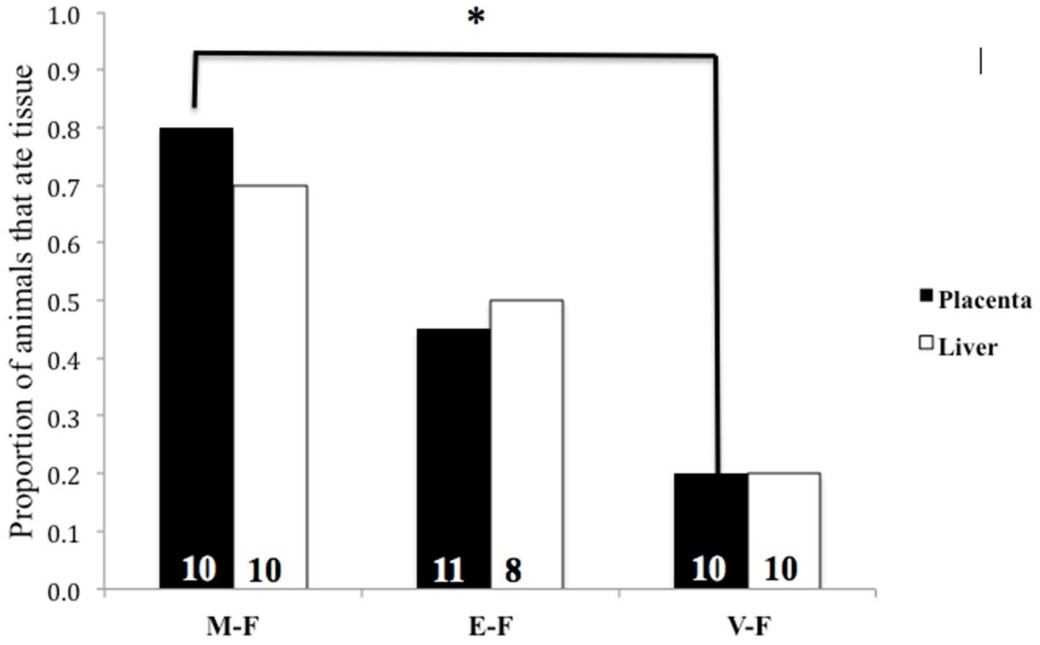
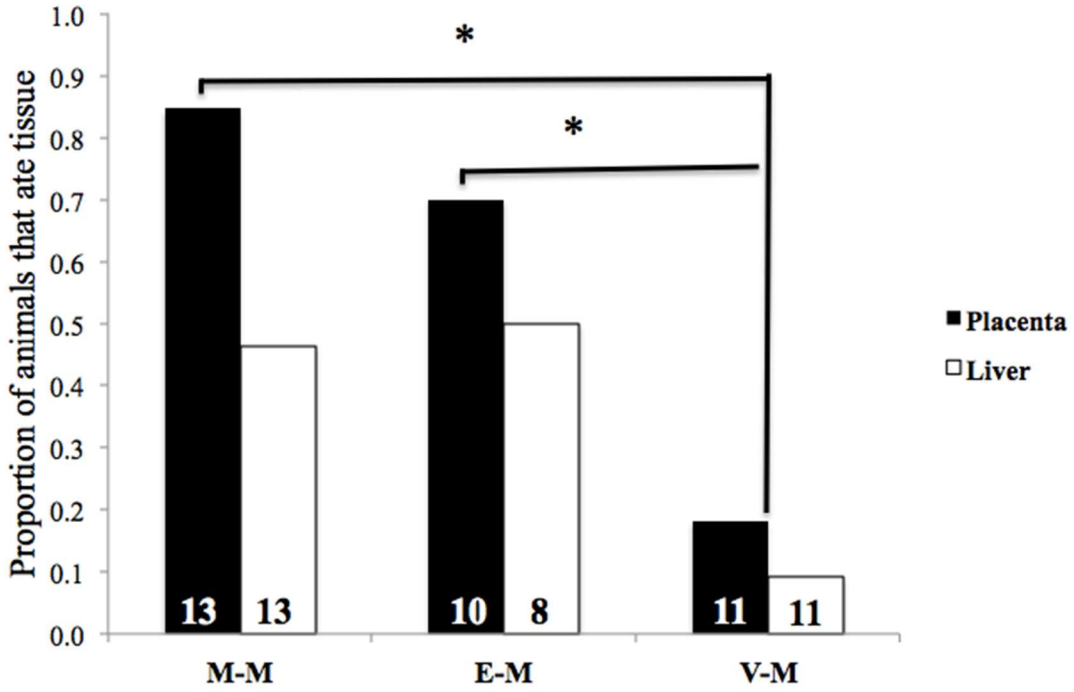


Figure1.2: Proportion of male California mice that ingested placenta (black bars) and liver (white bars) among multiparous (M-M), expectant (E-M), and virgin males (V-M). Numbers within bars represent sample sizes. * - P<0.016 (Fisher's Exact-Boschloo tests).



Chapter 2

Possible Effects of Placentophagia by Virgin Male California Mice (*Peromyscus californicus*)

Juan P. Perea-Rodriguez [1, 2], Trynke R. de Jong [3], Eric Kung [1], Nathan D. Horrell[1, 4] and Wendy Saltzman[1, 2, 4]

[1]Department of Biology, University of California, Riverside, [2]Evolution, Ecology, and Organismal Biology Graduate Program, University of California, Riverside, [3]Department of Behavioral and Molecular Neurobiology, University of Regensburg, Germany, [4] Graduate Program in Neuroscience, University of California, Riverside

Abstract

Ingestion of placenta by parturient females can lead to physiological changes that enhance maternal care and offspring survival. We hypothesized that similar effects of placentophagia might occur in males of a biparental mammalian species. To test this hypothesis, we administered either conspecific placenta in oil or oil alone to sexually inexperienced adult male California mice via oral gavage. One, 7 or 24 hours later, each male underwent a behavior test with either an unfamiliar pup or a control object (marble). Brains were collected for quantification of neural activation (Fos-immunoreactivity: Fos-ir) in areas involved in paternal care and/or stress/anxiety (bed nucleus of the stria terminalis, medial preoptic area, amygdala). At 7 h post-gavage, placenta treatment decreased latencies to approach both pups and marbles, compared to oil treatment ($p=0.05$). Placenta-treated males also showed significantly lower Fos-ir in the dorsal bed nucleus of the stria terminalis, irrespective of stimulus type, compared to oil-treated controls, both 1 h ($p=0.04$) and 7 h ($p=0.05$) after treatment. Fos-ir in the remaining brain regions studied did not differ between treatments at any time point. These results suggest that placentophagia by males may result in transient changes in behavior and in neural responsiveness of the dorsal bed nucleus of the stria terminalis, potentially associated with reduced neophobia. Thus, placentophagia might facilitate interactions between males and novel stimuli, including pups.

Keywords: Placentophagia, parental care, medial preoptic area, paraventricular nucleus, bed nucleus of the stria terminalis, amygdala

Introduction

Placentophagia, or ingestion of the afterbirth, is commonly performed by parturient females in most eutherian species, with some exceptions (e.g., pinnipeds and cetaceans: Kristal, 1980). Although humans do not typically eat placenta (Young & Benyshek, 2010), this practice has been performed increasingly in modern Western societies (Coyle et al., 2015, Marraccini & Gorman, 2015). The functional significance of placentophagia is unclear, but proposed explanations include avoiding predators or pathogens and meeting general or specific nutritional demands (reviewed by Kristal, 1980, Kristal et al., 2012). In rats (*Rattus norvegicus*) and cows (*Bos spp.*) placentophagia enhances opioid-mediated analgesia (Hoey et al., 2011, Kristal, 1991, Kristal et al., 2012, Pinheiro-Machado et al., 1997), which may occur as soon as 5 minutes after ingestion, highlighting the rapid effects of the biologically active component(s) in the afterbirth (Doer & Kristal, 1989). Decreased pain sensitivity during parturition may facilitate labor and increase offspring survival, as neonates may be expelled more quickly (Kristal, 1991). Additionally, ingestion of placenta by amniotic fluid in adult, sexually naïve rats enhances the stimulatory effect of intracerebroventricular morphine treatment on maternal sensitization (Neumann et al., 2009).

Placentophagia may be able to enhance offspring-directed care via specific physiological changes, as the placenta produces various steroid and protein hormones involved in social behavior (Malassine et al., 2003). For example, parturient rats allowed to ingest placenta while giving birth show increased plasma prolactin concentrations 1 day post-partum, as well as decreased plasma progesterone levels 6-8 days post-partum,

when compared to parturient females not allowed to ingest placenta (Blank & Friesen, 1987). These hormonal changes may increase milk production and/or enhance maternal behavior (Blank & Friesen, 1987, Siegel & Rosenblatt, 1978). The few studies that have addressed the effects of placentophagia in human mothers suffer from several methodological problems (e.g., absence of a control group, no mention of whether ingested placenta was their own: Coyle et al., 2015, Marraccinni & Gorman, 2015). Overall, however, these studies suggest that ingestion of dried placenta during the initial 2 weeks after parturition might increase milk output and might change the nutritional composition of milk compared to mothers that do not eat placenta (Hammett, 1918, Hammett & McNeile, 1917a, 1917b, Soyková-Pachnerová et al., 1954). These data suggest that placentophagia results in distinct physiological and behavioral changes in mothers, which in turn modulates maternal care and potentially enhance offspring development and survival.

In several biparental (i.e., both males and females care for their young) mammals, males, in addition to females, sometimes ingest placenta during the birth of their offspring. Among primates, placentophagia by males has been observed in the common marmoset (*Callithrix jacchus*: T. E. Ziegler, pers. comm.), cotton-top tamarin (*Saguinus oedipus*: T. E. Ziegler, pers. comm.), and silvery marmoset (*C. argentata*: J. A. French, pers. comm.), as well as in some human populations (Coyle et al., 2015, Marraccini & Gorman, 2015). In biparental rodents, placentophagia by males has been reported in dwarf hamsters (*Phodopus campbelli*: Gregg & Wynne-Edwards, 2005, Jones & Wynne-Edwards, 2000), California mice (*Peromyscus californicus:* Lee & Brown, 2002, Perea-

Rodriguez & Saltzman, 2014), and prairie voles (*Microtus ochrogaster*: K.L. Bales, pers. comm.). In the uniparental Siberian hamster (*Phodopus sungorus*) males ingest experimentally presented placenta only if they are present at the birth of their pups (Gregg & Wynne-Edwards, 2006). Studies in dwarf hamsters and California mice suggest that males, similar to females, respond differently to placenta depending on their reproductive condition. In these two species, males are more likely to ingest placenta when housed with their gestating mates and when they become fathers than when they are sexually inexperienced (Gregg & Wynne-Edwards, 2005, Perea-Rodriguez & Saltzman, 2014). Furthermore, male rats have been reported to ingest placenta after frequent exposure to it (Abbott et al., 1991).

These findings suggest that in at least some biparental mammals, males become attracted to placenta during their mates' pregnancy and may commonly ingest placenta during the birth of their young. Still unknown, however, are the potential behavioral and/or physiological changes that males undergo as a consequence of ingesting placenta, and how these changes influence the males' responses towards their young. We predicted that the potential (neuro)endocrine changes resulting from ingestion of placenta lead to changes in both behavioral and neural responses to pup-related stimuli (Brunton & Russell, 2008) in male California mice, a monogamous, biparental rodent in which fathers engage in all the same parental behaviors as mothers, and at frequencies; fathers even exhibit nursing postures, although they do not lactate (Dudley 1974, Gubernick & Alberts, 1987). We monitored behavioral and neural responses to a pup or a novel object (a pup-sized marble) after oral administration of placenta. To do so, we analyzed the

presence of the protein Fos, a product of the c-Fos immediate-early gene that is commonly used as a marker of neuronal activity (Hoffman & Lyo, 2002), in key brain areas involved in paternal care.

As a first step in characterizing the effects of placentophagia in males, we used adult virgin males because they are highly variable in their behavioral responses to pups, whereas virtually all fathers show pronounced, rapid-onset paternal behavior (de Jong et al., 2009, de Jong et al., 2012, Gubernick & Nelson, 1989). To identify both short- and long-term possible effects of placentophagia, we characterized behavioral and Fos-ir responses to a pup or novel object at 1, 7, and 24 hours after placenta administration. We predicted that mice treated with placenta would approach pups more rapidly, would spend more time engaging in caretaking behaviors, and would express more Fos-ir in brain areas positively linked to paternal care (ventral bed nucleus of the stria terminalis, medial preoptic area), as well as reduced Fos-ir in brain areas commonly activated by aversive stimuli (paraventricular nucleus of the hypothalamus, amygdala), compared to controls.

Methods

Animals

We used male California mice born and reared in our breeding colony at the University of California, Riverside and descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC). Mice were housed in standard, shoebox-style, polycarbonate cages (44 x 24 x 20 cm) containing aspen

shavings for bedding and cotton wool for nesting material, with *ad libitum* access to food (Purina Rodent Chow 5001) and water. Lighting was on a 14:10 light:dark cycle, with lights on from 05:00 until 19:00 h. Ambient temperature and humidity were kept at approximately 23°C and 70%, respectively. Mice were checked daily and weighed twice weekly, and cages were changed weekly.

Mice were weaned at 27-31 days of age and housed in same-sex groups of three or four age-matched individuals; these groups contained no more than two siblings from any one litter. As mice reached the age of sexual maturity (~90 days: Gubernick, 1988), male groups were divided into pairs of unrelated males.

Experimental Design

Virgin male mice were treated with either placenta or sesame oil via oral gavage (see below). Beginning 1, 7, or 24 h later, each mouse underwent a 1-h behavior test with either a 1- to 4-day-old pup or a control object - a pup-sized glass marble. Immediately following the behavior test (i.e., 2, 8 or 25 h after placenta or oil treatment), mice were euthanized and brains harvested for immunohistochemical analyses (see below). Each virgin male mouse was tested under a single treatment condition (placenta or oil), at a single time point (1, 7, or 24 h after gavage), and with a single test stimulus (pup or marble). At the time of testing, mice had never been exposed to pups (other than their own littermates) or marbles. The resulting sample sizes for each treatment, time point, and stimulus type are shown in Table 1.

Mice assigned to the *placenta* group were administered a single near-term placenta (from an unrelated female) homogenized in sesame oil. Mice in the *oil* group

were administered sesame oil alone. We administered placenta (or oil) via oral gavage because virgin male California mice are not likely to ingest placenta voluntarily (Perea-Rodriguez & Saltzman, 2014). Mice from the two treatments did not differ in age at the time of testing (placenta: 158.9 ± 4.3 days, mean ± SEM; oil: 162.9 ± 5.2 days; p=0.63, T=0.46, df=1; unpaired T-Test).

Placenta Collection

Placentas were collected from multiparous (2-7 previous litters) females 1-3 days prior to their estimated parturition date, determined by the date of their previous parturition and assessment of changes in female body mass based on measurements every 3-4 days. Fetuses were inspected visually to confirm that they were near-term, and immediately euthanized with an intraperitoneal injection (0.1mL) of pentobarbital sodium (Fatal-Plus: Vortech Pharmaceuticals, Dearborn, Michigan, USA). Placenta donors were euthanized using CO_2 inhalation, and placentas were removed and immediately stored at -70° C (Perea-Rodriguez & Saltzman, 2014).

Oral Gavage

Oral gavage was performed using a 5 cm length of Silastic® laboratory tubing (1.57 mm inside diameter x 2.41 mm outside diameter; Dow Corning, Copley, Ohio, USA) fitted onto an 18-gauge sterile needle; the needle's tip (~ 0.5 cm) had been filed off to avoid puncturing the tubing and injuring the animal. The needle was attached to a sterile 1 mL syringe containing either a single placenta (~0.4 g, and 0.1-0.2 mL in volume) homogenized in sesame oil (total volume: 0.5 mL) or 0.5 mL sesame oil alone. This volume was selected based on the size of the stomach and to minimize any discomfort to

the mice. We used oil as a vehicle because we anticipated that any hormonally mediated effects of placentophagia would likely be related to steroid hormones, as these hormones readily cross the blood-brain barrier and are biologically active follow ingestion; steroid hormones are hydrophobic and therefore oil-soluble.

Mice underwent oral gavage between 8:30 and 9:30 h. We treated animals in the morning because this is the time of day when California mice are most likely to give birth (within a few hours after lights-on; Lee & Brown 2002) and therefore to ingest placenta. Each male mouse was first placed alone into a clean isolation cage containing fresh bedding, food and water for 30 min. Placentas were thawed on ice and homogenized in 0.1-0.2 mL of sesame oil using a mortar, and pestle and collected using the sterile syringe, which was then attached to the 18-gauge needle fitted with the Silastic tubing; air bubbles were avoided as much as possible. Mice were lightly anesthetized using isoflurane (Minrad, Orchard Park, NY, USA) and held vertically as the tubing was carefully inserted into the esophagus and the contents of the syringe delivered over approximately 5-10 s. The recovery time from anesthesia was between 60 and 180 s, at which point animals were observed in their isolation cages for 10 min before being returned to the colony room. Mice remained in their isolation cages in the colony room until testing began 1, 7, or 24 h later.

Behavior Testing

Each animal underwent a behavior test in the colony room during the lights-on phase of the light:dark cycle, beginning at 09:30-10:30 h (1 h after oral gavage), 16:30-17:30 h (7 h after gavage), or 09:30-10:30 h the next day (24 h after gavage). At the outset of each

test a 1- to 4-day-old pup (no more closely related to the male than second cousin) or a pup-sized, oblong, glass marble was placed at the opposite end of the cage from the focal animal. Each mouse was exposed to its respective stimulus for 60 min before being euthanized for tissue collection (see below). Behavior tests were videotaped and the initial 20 minutes were later scored using JWatcher software (Blumstein & Daniel, 2007). Behaviors scored were latency to approach the pup or marble, duration of investigating (i.e., sniffing) pup, and duration of huddling + grooming pup (i.e., caretaking behavior). The experimenter scoring the videos was blind to the animals' treatment.

Brain Collection, Immunohistochemistry, and Fos-ir Quantification

Brains were harvested for immunohistochemical staining for c-Fos, a protein indicator of neuronal activation (Hoffman & Lyo, 2002), as previously described (de Jong et al., 2009). Immediately after each hour-long behavior test, the focal mouse was deeply anesthetized with 10% pentobarbital (Vortech, Dearborn, Michigan, USA; 0.5 mL, i.p.) and perfused transcardially, first with 0.1M phosphate-buffered saline (PBS) and subsequently with 4% paraformaldehyde (PFA). Brains were placed in 4% PFA for 1 h immediately after perfusion to further increase tissue robustness. After the additional fixation period, brains were removed from PFA and stored in 0.1M PBS until further processing. Brains were cryoprotected in 30% phosphate-buffered sucrose before being sliced into 30 μ sections on a cryostat set at -19°C. Prior to slicing, brains were embedded in optimal cutting temperature compound and frozen. Five series of brain sections were collected sequentially and stored in 0.1M PBS with 0.01% sodium azide until staining occurred.

After pre-incubation with PBS containing 0.1% bovine serum albumin and 0.3% Triton-X-100 (i.e., PBS-BT), slices were incubated in a 1:10,000 dilution of rabbit-anti-c-Fos antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS-BT overnight. The next day, after removal of excess antibody through a series of PBS washes, the slices were incubated with donkey-anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in a 1:1,500 dilution with PBS-BT for 90 min. Signaling was enhanced using ABC-vector (1:800 dilution in PBS-BT, Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) before being stained with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in 0.6% Tris-buffer.

Using fine brushes, stained slices were mounted onto glass slides coated with gelatin and chrome alum. Mounted slices were air-dried overnight, cleared using a range of alcohols, and embedded in Entellan New (EMS, Hatfield, PA, USA) before being coverslipped. Micrographs of stained and mounted brain slices were taken using a digital camera (Canon EOS 40D) attached to a microscope (Leica Leitz DMRB). Micrographs of the medial preoptic area (MPOA), the dorsal (dBST) and ventral (vBST) regions of the bed nucleus of the stria terminalis, the paraventricular nucleus of the hypothalamus (PVN), and the central (CeA) and basolateral (BLA) nuclei of the amygdala were taken for each brain (Figure 2.1). Because no brain atlas is available for *Peromyscus,* brain regions/nuclei of interest were located based on a standard atlas of the mouse brain (Paxinos & Franklin, 2004), as in previous studies (de Jong et al., 2009, de Jong et al., 2012).

ImageJ software (1.46r; National Institutes of Health, USA) was used to count the number of Fos-ir neurons in a 200 x 200 μm square in a representative area of neurons in each region. The person counting was unaware of the treatment or stimulus of each animal. Some of the brain sections were not usable due to problems during the sectioning or staining process, so these were excluded from the analyses. The final sample sizes are presented in the results.

Statistical Analyses

All statistical analyses were performed using R statistical software (R Core Team, 2014). Behavioral and immunohistochemical data were tested for normality using Shapiro-Wilk tests. Bartlett's tests were used to determine homogeneity of variance. Because data collection and immunohistochemical staining for the three time points were performed separately, data from each time point were analyzed independently. Normally distributed data (latency to approach stimuli, all Fos-ir data) were analyzed by 2-way ANOVAs, with treatment (placenta, oil) and stimulus (pup, marble) as factors. If a significant ($p \leq 0.05$) treatment x stimulus interaction was found, we performed post-hoc pairwise comparisons using Tukey's HSD tests. Non-normal data (duration of huddling + licking pup, duration of investigating pup) were analyzed using Mann-Whitney U tests to compare behavioral responses in placenta- vs. oil-treated mice.

Results

Behavioral Responses to Stimuli

Among the mice tested with a pup at each time point, the proportion that showed paternal

behavior (i.e., licking and/or huddling pup) did not differ between placenta- and oil-treated males (all p-values>0.50, Fisher's Exact test for each time point; Table 1.1). Additionally, placenta treatment did not affect the total duration of caretaking behavior (huddling + licking) that mice engaged in at any time point (1 h: p=0.50; 7 h: p=0.94; 24 h: p=0.45; Mann-Whitney U test for each time point; Figure 2.2). At 7 h post-gavage, placenta-treated mice approached their assigned stimuli more quickly than oil-treated mice (main effect of treatment: $F_{1, 25}$=4.22, p=0.05; 2-way ANOVA); however, this effect did not differ between males tested with pups and those tested with marbles (main effect of stimulus: p=0.15; treatment x stimulus interaction: p=0.43). Latencies to approach pups or marbles did not differ significantly between placenta- and oil-treated mice at either of the other time points (1 h: main effect of treatment: p=0.54; main effect of stimulus: p=0.50; treatment x stimulus interaction: p=0.66; 24 h: main effect of treatment: p=0.63; main effect of stimulus: p=0.71; treatment x stimulus interaction: p=0.56; 2-way ANOVA for each time point). Finally, treatment had no effect on the total duration of time mice spent sniffing pups at any of the time points (1 h: p=0.48; 7 h: p=1.00; 24 h: p=0.30; Mann-Whitney U test for each time point).

Neural Responses to Stimuli

In general, Fos-ir in the brain areas investigated was lower in mice treated with placenta than in those treated with oil, although in most cases the difference was not statistically significant (Table 2.2). Treatment with placenta significantly altered neural responses to stimuli in the dBST at both the 1 h and 7 h time points. Placenta-treated mice tested 1 h after oral gavage had significantly lower Fos-ir in the dBST regardless of stimulus,

compared to oil-treated controls (main effect of treatment: $F_{1, 20}$=4.51, p=0.04; 2-way ANOVA; Table 2, Figure 2.3). At this time point, Fos-ir in the dBST was not influenced by stimulus type (main effect of stimulus: p=0.54) nor by an interaction between treatment and stimulus (p=0.87).

At the 7 h time point, placenta-treated mice still showed a reduction in Fos-ir in the dBST compared to oil-treated controls (main effect of treatment: $F_{1, 18}$=4.13, p=0.05), and this effect differed between males exposed to a pup and those exposed to a marble (treatment x stimulus interaction: $F_{1, 18}$=7.33, p=0.01; 2-way ANOVA). Among placenta-treated mice, those exposed to a pup 7 h after gavage showed a reduction in dBST Fos-ir compared to males exposed to a marble, but this reduction was not statistically significant (p=0.06, Tukey's HSD test); no such effect was seen in oil-treated animals (p=0.69). At 24 h post-treatment, Fos-ir in the dBST was not significantly influenced by a main effect of treatment or stimulus, or by an interaction between these two factors (all p-values >0.33).

In contrast to Fos-ir in the dBST, Fos-ir in the MPOA, vBST, PVN, BLA, and CeA was not significantly affected by treatment (all p-values >0.07; Table 2.2, Figure 2.3). One hour after gavage, Fos-ir in both the BLA and CeA was differentially affected by exposure to pups vs. marbles: mice exposed to a pup showed higher Fos-ir in the BLA (main effect of stimulus: $F_{1, 20}$=4.60, p=0.04; 2-way ANOVA) and CeA (main effect of stimulus: $F_{1, 20}$=5.71, p=0.02; 2-way ANOVA), compared to mice exposed to a marble. However, neither of these effects differed between placenta- and oil-treated animals (p-values >0.12).

Discussion

In this study, we aimed to identify possible behavioral and neural consequences of placentophagia by males in a monogamous, biparental species. Specifically, we sought to investigate the possible role of placentophagia in facilitating pup-directed care in the California mouse, as males of this species ingest placenta during the birth of their offspring (Lee & Brown, 2002, Perea-Rodriguez & Saltzman, 2014) and engage in extensive paternal behavior (Gubernick & Alberts, 1987), and to identify neural correlates of these behavioral effects.

Our major finding was that 7 hours after placenta treatment, virgin male California mice showed reduced latencies to approach pups and marbles, compared to oil-treated mice. In addition, placenta treatment resulted in reduced pup- and/or novel-object (marble)-induced activation (Fos-immunoreactivity) of the dorsal region of the bed nucleus of the stria terminalis (dBST) 1 and 7 h after treatment. Taken together, these findings indicate that ingesting placenta reduces responsiveness of the dBST as rapidly as within 1 h and for as long as 7 h or more, which may be associated with reduced latencies to approach pups or other novel stimuli. On the other hand, ingestion of placenta did not alter paternal behavior or neural activity in other brain regions, including the PVN, BLA, CeA, vBST, and, most strikingly, the MPOA, which has been implicated in paternal behavior in California mice and other biparental rodents; Bales & Saltzman, 2016).

The BST is a limbic forebrain structure that has been linked to paternal care, stress, anxiety, and aggression in California mice and other species (Bester-Meredith & Marler, 2003, Crestani et al., 2008, Davis & Marler, 2004, Davis et al., 2010, de Jong et

al., 2009, Trainor et al., 2010), and neurochemical changes in the BST may alter an animal's behavioral response to unpredictable, threatening, and aversive stimuli (i.e., unconditioned fear) (Walker & Davis, 1997). In rodents the BST contains dorsal and ventral regions that differ in their electrophysiological and neurochemical properties (Egli & Winder, 2003, Frazier et al., 2006); however, both of these regions show increased Fos-ir under stressful conditions (Di Bonaventura et al., 2014).

Importantly, in two biparental species, prairie voles and California mice, fatherhood results in changes in stress reactivity and anxiety-like behaviors, respectively, suggesting that males undergo changes in how they perceive potentially aversive or novel stimuli with changes in reproductive state or reproductive experience (Bardi et al., 2011, Chauke et al., 2012, Lieberwirth et al., 2013). In the same two species, paternally responsive males have increased Fos-ir in the medial posteromedial and medial BST after exposure to pups (de Jong et al., 2009; Kirkpatrick et al., 1994). Moreover, in the biparental Mandarin vole (*Microtus mandarinus*) and California mouse, male parental responsiveness is associated with neuroendocrine changes in the BST. Mandarin vole fathers show reduced density of estrogen receptor alpha (ERα) in the dorsal BST compared to non-fathers (Song et al., 2010), whereas parentally responsive virgin males show increased ERα densities in the BST, compared to non-paternally responsive males (Li et al., 2015). California mouse fathers have decreased mRNA expression of receptors for oxytocin, vasopressin (V1a) and progesterone in the oval nucleus of the BST and lateral posterior BST compared to virgin males (Perea-Rodriguez et al., 2015). In contrast, ERα-ir in the BST does not differ between fathers and non-fathers of the

biparental Djungarian hamster (Timonin et al., 2008), and increased expression of ERα via viral vector in the BST of male prairie voles does not alter pup-directed care. Thus, the role of the BST in paternal care, if any, is not yet known.

Studies on the consequences of placentophagia, although scarce, suggest that placentophagia by mothers may trigger behavioral and physiological changes that positively affect their offspring (e.g., Abbott et al., 1991, Blank & Friesen, 1987, González-Mariscal et al., 1998). In the case of males, a single study on rats, which are uniparental, showed that virgin males experience hypoalgesia after ingesting placenta (Abbott et al., 1991). Although placentophagia did not enhance pup-directed care in our study, it decreased males' latencies to approach both pups and marbles 7 h post-treatment and led to changes in neural activity in (virgin) males. Therefore, we propose that placentophagia might influence an individual's response to novel objects in general (i.e., neophobia) rather than to pups specifically. If placentophagia has similar effects in new fathers to those in virgin males, it might facilitate contact between males and neonates in the post-partum period by reducing fathers' anxiety or fearfulness. This effect, in combination with other cues from the pregnant/parturient mate and/or hormonal changes in the fathers, could potentially lead to an increased propensity to provide caretaking behaviors to neonates.

Our data indicate that the behavioral and neuronal effects of placenta ingestion are short-lived: changes in Fos-ir were detected as early as 1 h post-treatment and persisted for at least 7 h, but no changes in behavior or Fos-ir were seen 24 h after placenta treatment. It is not known which of the many biologically active products of placenta are

responsible for these short-term changes. Placenta contains both peptides and steroid hormones involved in paternal care (e.g., estrogens, placental lactogens, oxytocin: Malassine et al., 2003, Saltzman & Ziegler, 2014), all of which can act rapidly through membrane-receptor-mediated, non-genomic effects (Gimpl & Fahrenholz, 2001, Kelly & Levin, 2001) or by changes in enzymatic activity (Cornil & Charlier, 2010). In addition, steroid hormones can have slower, more prolonged effects mediated by intracellular receptors and changes in gene expression (Cornil & Charlier, 2010). Steroid hormones are of special interest in the context of this study as they are expected to remain undigested after ingestion and readily cross the blood-brain barrier (Pardridge, 1995). Alternatively, behavioral and neural effects of placentophagia might be mediated not by placental hormones but by nonspecific factors such as nutrients found in placenta. We did not evaluate this possibility by using a control substance such as liver, another highly vascularized tissue, because we wanted to identify consequences of placentophagia *per se* regardless of specific mechanism. In male and female California mice, however, changes in reproductive status affect the propensity to eat placenta but not liver (Perea-Rodriguez & Saltzman, 2014).

Some important caveats should be kept in mind when interpreting the results of this study. First, although we found behavioral and neural changes after placenta ingestion in virgin males, it is unknown whether comparable changes would occur in fathers. Mating, pair bonding, and/or fatherhood can result in neural, neuroendocrine, and behavioral changes in males of biparental species (Bales & Saltzman, 2016, Saltzman & Ziegler, 2014), which might influence how fathers respond to biologically active

components of placenta. Second, although c-Fos expression has been linked to changes in cellular activity, this is not always the case; c-Fos may or may not be expressed when neurons undergo changes in gene expression or electrical activity (Hoffman & Lyo, 2002). Third, the sample sizes in this study were relatively small. Finally, the oral gavage procedure by which we administered placenta eliminated possible effects that placenta and amniotic fluid may have via olfactory or accessory olfactory pathways, and the oil preparation used may have limited absorption of some of the chemicals found in placenta and amniotic fluid.

In conclusion, this is the first study to investigate the functional consequences of male placentophagia in a biparental mammal. We found that although ingestion of placenta did not alter the expression of paternal behavior by virgin males, it transiently reduced Fos-ir in the dorsal region of the bed nucleus of the stria terminalis after exposure to pups and other novel objects, as well as males' latencies to approach novel stimuli. These findings suggest that placentophagia by males of biparental species may increase their propensity to interact with novel stimuli, potentially including neonates.

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Table 2.1: Sample sizes per time point and stimulus. Bold numbers represent the number of male mice tested with a pup that showed pup-directed care (huddling and/or grooming pup).

1 h Post-treatment		
	Stimulus	
Treatment	Pup	Marble
Placenta	8; **4**	6
Oil	7; **2**	6
7 h Post-treatment		
	Stimulus	
Treatment	Pup	Marble
Placenta	7; **5**	6
Oil	7; **5**	5
24 h Post-treatment		
	Stimulus	
Treatment	Pup	Marble
Placenta	9; **6**	7
Oil	8; **4**	7

Table 2.2: Number of Fos-positive neurons following exposure to a pup or control object (marble) at each of three time points after treatment with placenta or oil. Data were analyzed using 2-way ANOVAs. Means, standard errors, and sample sizes are shown, as well as P-values for main effects of treatment (upper P-value in each cell) and treatment x stimulus interactions (lower p-value in each cell). P-values ≤ 0.05 are shown in bold. MPOA – medial preoptic area of the hypothalamus, dBST – dorsal bed nucleus of the stria terminalis, vBST – ventral bed nucleus of the stria terminalis, PVN – paraventricular nucleus of the hypothalamus, BLA – basolateral amygdala, CeA – central nucleus of the amygdala.

		1h Post-Treatment			7h Post-Treatment			24h Post-Treatment		
Brain Area	**Stimulus**	Oil	Placenta	P-value	Oil	Placenta	P-value	Oil	Placenta	P-value
MPOA	Marble	14.75 ± 4.73 n=6	22.75 ± 6.10 n=6	0.93	10.10 ± 2.35 n=5	13.08 ± 3.21 n=6	0.46	15.60 ± 5.71 n=5	13.14 ± 2.81 n=7	0.65
	Pup	27.83 ± 5.02 n=6	19.00 ± 3.25 n=6	0.10	7.33 ± 1.20 n=6	7.91 ± 2.85 n=6	0.64	17.62 ± 4.92 n=8	13.00 ± 2.49 n=8	0.78
dBST	Marble	36.00 ± 5.48 n=6	27.58 ± 2.95 n=6	**0.04**	24.80 ± 5.42 n=6	26.91 ± 3.80 n=6	**0.05**	18.00 ± 5.83 n=5	27.57 ± 6.58 n=7	0.75
	Pup	34.08 ± 4.40 n=6	24.25 ± 3.93 n=6	0.87	30.50 ± 2.86 n=6	12.80 ± 1.49 n=5	**0.01**	25.87 ± 5.56 n=8	24.5 ± 6.12 n=8	0.33
vBST	Marble	12.33 ± 1.97 n=6	10.91 ± 1.43 n=6	0.78	8.60 ± 1.81 n=5	11.66 ± 2.11 n=6	0.91	9.87 ± 1.57 n=4	11.25 ± 1.25 n=2	0.78
	Pup	11.33 ± 1.92 n=6	14.25 ± 1.27 n=6	0.27	10.08 ± 1.47 n=5	7.10 ± 0.92 n=5	0.09	10.33 ± 1.45 n=3	12.2± 3.10 n=5	0.27
PVN	Marble	37.60 ± 4.61 n=5	27.7 ± 2.22 n=6	0.07	22.90 ± 2.14 n=5	27.36 ± 4.01 n=6	0.41	24.85 ± 3.54 n=7	23.50 ± 5.50 n=6	0.72
	Pup	53.83 ± 11.03 n=6	34.54 ± 6.65 n=6	0.37	20.58 ± 6.56 n=6	24.16 ± 5.06 n=6	0.93	26.5 ± 5.69 n=8	35.42 ± 5.38 n=7	0.31
BLA	Marble	24.83 ± 2.52 n=6	24.00 ± 4.52 n=6	0.31	21.90 ± 5.08 n=5	20.41 ± 3.23 n=6	0.85	21.33 ± 3.17 n=6	29.00 ± 5.95 n=7	0.60
	Pup	39.91 ± 5.43 n=6	30.41 ± 6.63 n=6	0.39	21.08 ± 5.85 n=6	20.90 ± 1.17 n=5	0.88	33.60 ± 3.60 n=5	36.66 ± 11.02 n=6	0.69

Figure 2.1: Brain nuclei where Fos-immunoreactivity was determined.

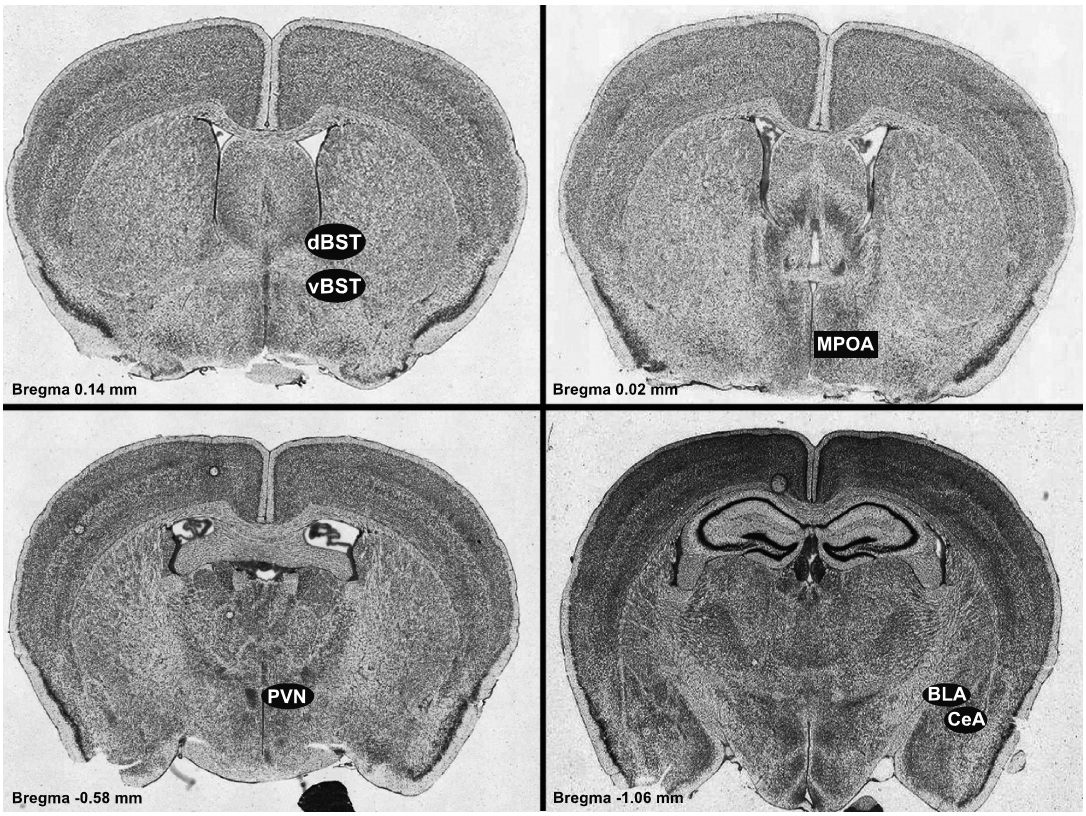


Figure 2.2: Behavioral responses to a 1- to 4-day-old pup by virgin male California mice 1, 7, or 24 after treatment with oil or placenta. Bars represent medians, and error bars represent 1st and 3rd quartiles. Asterisks indicate significant differences between treatments ($p \leq 0.05$).

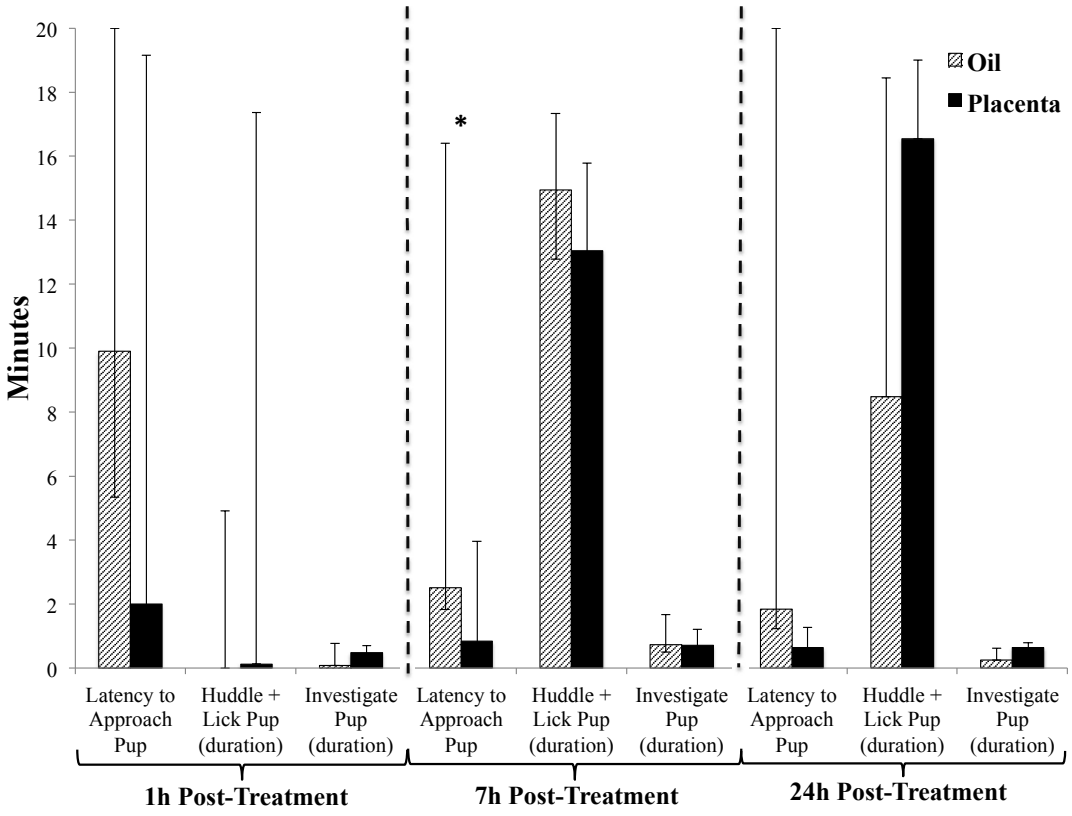
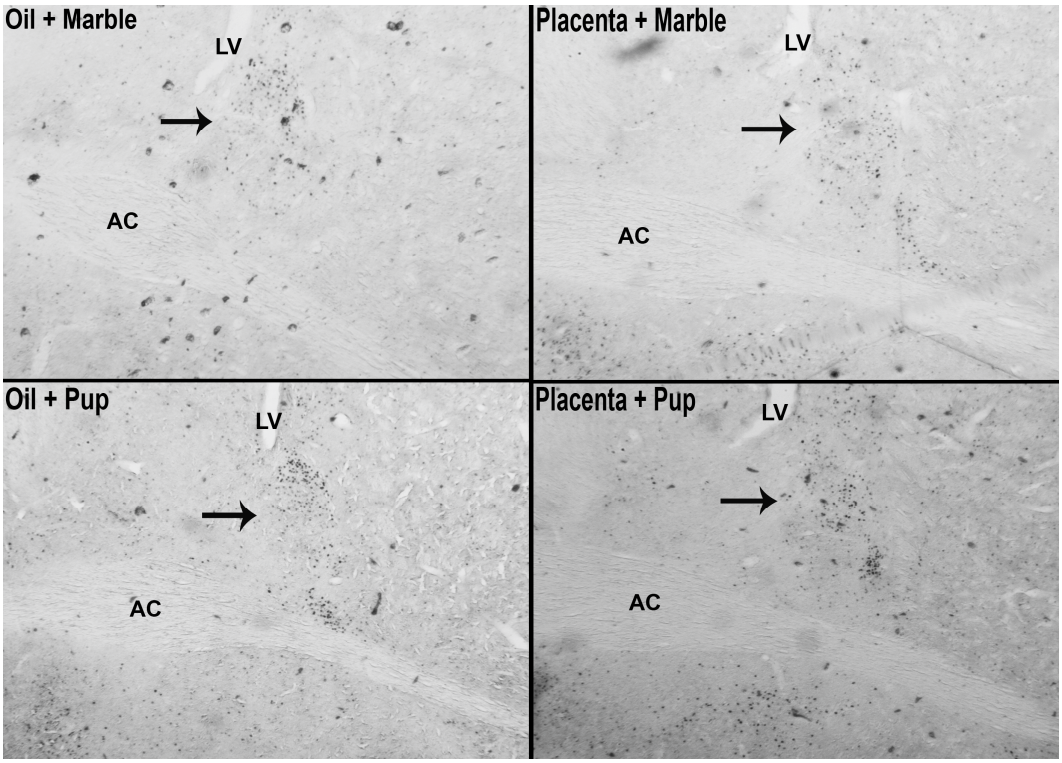


Figure 2.3: Representative photomicrographs of Fos labeling in the dorsal bed nucleus of the stria terminalis of oil- and placenta-treated virgin male California mice. Fos is stained blue-black (nuclear staining), as indicated by the arrows. AC: anterior commissure; LV: lateral ventricle.



Chapter 3

Effects of Placentophagia by Male California Mice (*Peromyscus californicus*) on Pain Sensitivity, Exploratory Behavior, and Parental Care

Juan P. Perea-Rodriguez [1, 2], Joel Raqueno [1], Meng Zhao[1, 2], Wendy Saltzman[1, 2,]

[1]Department of Biology, University of California, Riverside, [2]Evolution, Ecology, and Organismal Biology Graduate Program, University of California, Riverside

Abstract

Placentophagia by mothers can lead to changes in pain sensitivity and behavioral responses to newborns. In some biparental rodents, such as the California mouse (*Peromyscus californicus*), males, in addition to females, ingest placenta when their pups are born. Interestingly, males first become attracted to placenta when cohabitating with their gestating mate, and virgin male California mice administered placenta have reduced latencies to approach novel objects (i.e., neophobia) compared to males given oil vehicle. Still unknown is whether placentophagia can modulate pain sensitivity and anxiety-like behaviors, and how the effects of placentophagia may be influenced by a male's reproductive experience. Thus, we orally administered either placenta or oil vehicle to male California mice from three reproductive conditions (first-time fathers, first-time expectant fathers, and virgin males) and tested their pain sensitivity 1 hour later, as well as their exploratory behavior and paternal responsiveness in an open field 4 hours post-treatment. We found that placenta-treated males, independent of reproductive condition, traveled significantly longer distances in the open field than males treated with oil. Additionally, fathers had shorter latencies to care for pups (i.e., huddling, licking or carrying pups), and spent more time engaging in these behaviors, compared to age-matched expectant fathers and virgin males. These findings indicate that male California mice undergo changes in their exploratory behavior when they ingest placenta.

Keywords: Placentophagia, California mice, pain sensitivity, open-field test, exploratory behavior, paternal care

Introduction

Female mammals typically consume placenta while giving birth, with some exceptions (e.g., marine mammals, humans, camelids: Arendt et al., in revision). The functional significance of this behavior is unclear, but popular explanations include (a) general hunger (i.e., many parturient females become aphagic before labor and are motivated to eat highly nutritional placenta during or after parturition), (b) specific hunger (i.e., mothers lack a specific hormone or factor that is found in placenta, and replenish it through placentophagia), and (c) predator/pathogen avoidance (Kristal, 1980). Few of these or the other proposed hypotheses have been tested formally (Kristal, 1980). Regardless of the ultimate explanation for placentophagia, some female mammals change their behavioral response to placenta with changes in their reproductive condition. Specifically, females' response to placenta transitions from aversion when they are sexually inexperienced to attraction during late pregnancy and/or with birthing experience (Kristal et al., 1980, 2012). This behavioral transition has been reported in rats (*Rattus norvegicus*) house mice (*Mus musculus*) (Kristal, 1980), California mice (*Peromyscus californicus*; Perea-Rodriguez & Saltzman, 2014), Siberian hamsters (*Phodopus sungorus*; Gregg & Wynne-Edwards, 2006), dwarf hamsters (*P. campbelli*; Gregg & Wynne-Edwards, 2005, 2006), rabbits (*Oryctolagus cuniculus L.*; Melo & Gonzales-Mariscal, 2003), and sheep (*Ovis aries*) (Levy et al., 1983).

Importantly, after ingesting placenta, mothers undergo specific endocrine changes that can affect maternal responsiveness. For example, parturient rats allowed to ingest

placenta during parturition showed increased plasma prolactin levels one day post-partum, as well as decreased plasma progesterone levels 6-8 days post-partum, when compared to parturient females not allowed to ingest placenta (Blank & Friesen, 1980). Similarly, lactating rats that ingested conspecific placenta once per day showed significantly lower progesterone levels on day 5 of this regime when compared to controls (Blank & Friesen, 1980). Blockade of prolactin in non-pregnant female rats, which normally are not maternally responsive, abolished the subsequent onset of maternal care (Bridges & Ronsheim, 1990), whereas treatment with progesterone has been shown to delay the onset of maternal care in female rats (Siegel & Rosenblatt, 1975); thus, the hormonal changes elicited by placentophagia in rats may promote the expression of maternal care. Additionally, ingestion of placenta and amniotic fluid in adult, sexually naïve rats enhances the stimulatory effect of intracerebroventricular morphine treatment on maternal sensitization (Neumann et al., 2009). Placentophagia also increases opioid-mediated analgesia in female rats through opioid- enhancing factors found in placenta, even when placenta comes from a species that does not normally ingest placenta (i.e., humans, dolphins) (Kristal, 1991). These data suggest that placentophagia may result in distinct physiological changes in females, which may positively mediate several aspects of maternal care.

In several biparental mammals (i.e., both males and females care for their offspring), males also ingest placenta during the birth of their infants. In primates, for instance, male placentophagia has been seen in the common marmoset (*Callithrix jacchus*; T.E. Ziegler, pers. comm.), the cotton-top tamarin (*Saguinus oedipus*; T.E.

Ziegler, pers. comm.) and the silvery marmoset (*C. argentata*; J.A. French, pers. comm.). Among biparental rodents, male placentophagia has been reported in dwarf hamsters (Jones & Wynne-Edwards, 2000), California mice (Lee & Brown, 2002), and prairie voles (*Microtus ochrogaster*; K.L Bales, pers. comm.). Intriguingly, in the uniparental Siberian hamster, males will ingest experimentally presented placenta only if they were present at the birth of their first litter of pups (Gregg & Wynne-Edwards, 2006), and male rats are more likely to ingest placenta after repeated exposure to it (Kristal, 1991). Moreover, studies in the dwarf hamster and the California mouse show that, similar to females, males may respond differently to placenta depending on their reproductive condition. In these two species, males are more likely to ingest placenta when their mate is pregnant. What is still unknown is whether placentophagia by fathers elicits (neuro)endocrine and/or behavioral changes that may affect paternal responsiveness towards their newborn offspring.

Only two studies have investigated physiological and/or behavioral changes in males after ingestion of placenta, one in the uniparental rat and another in the biparental California mouse; both studies suggest that males can undergo physiological and/or behavioral changes after ingesting placenta. Male rats, similar to females, experience an increase in opioid-mediated analgesia, suggesting that ingestion of placenta and/or amniotic fluid may modify opioid signaling pathways (Kristal, 1991). Adult, virgin male California mice administered placenta homogenized in sesame oil via oral gavage showed decreased latencies to approach novel stimuli (i.e., an unrelated pup or a pup-sized-marble), as well as decreased neural activity (i.e., Fos expression: Hoffman & Lyo,

2002) in the dorsal bed nucleus of the stria terminalis 1 and 7 hours post-ingestion, compared to virgins treated with sesame oil alone (Perea-Rodriguez et al., in revision). However, no changes in caretaking behaviors were seen as a result of placenta treatment (Perea-Rodriguez et al., in revision). These data indicate that placentophagia may bring about a reduction in males' latency to approach and interact with novel stimuli, including pups, suggesting a reduction in anxiety (i.e., neophobia) and/or stress-related responses to novelty. Therefore, placentophagia may regulate how first-time fathers behave when they first encounter their pups, and thus may be one of the factors regulating the onset of paternal care in the California mouse. Consistent with this possibility, recent work on California mice suggests that fathers may be less anxious than non-fathers (Hyer et al. 2016), and that anxiety-related neural and behavioral measures correlate negatively with measures of paternal responsiveness (de Jong et al. 2012, Chauke et al. 2012).

No study has tested the effects of placentophagia on males' pain sensitivity in a biparental species. We hypothesized that placenta ingestion by males would decrease their pain sensitivity, as well as their anxiety-like behavior, potentially leading to changes in paternal responsiveness. To test this hypothesis, we evaluated pain-sensitivity, exploratory behaviors in an open field, and paternal responsiveness in adult male California mice that were treated orally with either conspecific placenta or oil vehicle. Because other reproduction-related stimuli, e.g., from mating or cohabitation with a pregnant female, might influence males' responses to placentophagia, we compared these effects in virgin males, new fathers, and first–time expectant fathers.

Methods

Animals

We used male California mice born and reared in our breeding colony at the University of California, Riverside and descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC). Mice were housed in standard, shoebox-style, polycarbonate cages (44 x 24 x 20 cm) containing aspen shavings for bedding and cotton wool for nesting material, with *ad libitum* access to food (Purina Rodent Chow 5001) and water. Lighting was on a 14:10 light:dark cycle, with lights on from 05:00 until 19:00 h. Ambient temperature and humidity were kept at approximately 23°C and 70%, respectively. Mice were checked daily and weighed twice weekly, and cages were changed weekly.

Mice were weaned at 27-31 days of age and housed in same-sex groups of three or four age-matched individuals; these groups contained no more than two siblings from any one litter. As mice reached the age of sexual maturity (~90 days: Gubernick, 1988), males and females were placed into same- or opposite-sex pairs of unrelated (see below).

Experimental Design

Male California mice were randomly assigned to one of the following reproductive conditions: sexually inexperienced males (i.e., virgins), first-time expectant fathers, and first-time fathers. *Virgins* (n=17) were paired with a same-sex, age-matched, unrelated cage mate from their initial group of 3-4 animals; both mice were used as experimental subjects. *Expectants* (n=16) and *fathers* (n=16) were paired with an age-matched female.

Paired males and females were no more closely related than second cousins. All mice were weighed twice weekly throughout the study, and pregnancies were monitored by body-mass changes in females.

One to five days after the birth of pups to a breeding pair, that female's mate (*father*) and 1-2 time-matched *expectants* and *virgins* underwent treatment and testing. Half of the males from each reproductive condition were administered a fresh, near-term placenta (~0.4 grams) from an unrelated, conspecific female, homogenized in sesame oil, whereas the remaining half of the males in each reproductive condition were administered oil alone (controls) via oral gavage. One hour after treatment each mouse was given a pain-sensitivity test, followed by exploratory-behavior and paternal-responsiveness tests 4 hours post-treatment (see below).

Placenta Procurement and Administration

Near-term placentas were collected as previously described (Perea-Rodriguez & Saltzman, 2014) from the first-time gestating females cohabitating with males from the *expectant* condition. Approximate parturition dates were determined by the presence of a sharp weight increase towards the last week of the gestation period (unpub. data). Near-term pups are also noticeable on the ventrolateral abdominal area of gestating mothers, and mothers' nipples increase in volume (unpub. obs). Near-term gestating females were euthanized by CO_2 inhalation, and their uterus was immediately dissected out and placed on a clean petri dish. Each individual fetus and its placental membranes were then freed from the uterine tissue using microscissors and forceps. The placenta was detached from

the fetus and placed into a 1 mL microcentrifuge tube, homogenized with 0.1 - 0.2 mL of sesame oil, and placed on ice. Fetuses were quickly euthanized by an intra-peritoneal injection of 0.1 mL of pentobarbital (Fatal-Plus: Vortech Pharmaceuticals, Dearborn, Michigan, USA).

Oral gavage was performed using a 5 cm length of Silastic® laboratory tubing (1.57 mm inside diameter x 2.41 mm outside diameter; Dow Corning, Copley, Ohio, USA) fitted onto an 18-gauge sterile needle; the needle's tip (~ 0.5 cm) had been filed off to avoid puncturing the tubing and injuring the animal. The needle was attached to a sterile 1 mL syringe containing either a single placenta (~0.4 g, and 0.1-0.2 mL in volume) homogenized in sesame oil (total volume: 0.5 mL) or 0.5 mL sesame oil alone.

Between 08:30 and 10:00 h on the morning of testing, each mouse was isolated in a clean cage containing bedding, food, and water. Mice underwent oral gavage 30-180 minutes after being isolated, in a procedure room adjacent to the colony room. Placentas were harvested between 08:00 and 09:30 h. Mice were treated in the morning because this is the time of day when California mice are most likely to give birth (within a few hours after lights-on; Lee & Brown 2002) and therefore to ingest placenta. Mice were lightly anesthetized using isoflurane (Minrad, Orchard Park, NY, USA) and held vertically as the tubing was carefully inserted into the esophagus and the contents of the syringe delivered over approximately 5-10 s. Mice were returned to their isolation cages for recovery. The recovery time from anesthesia was between 60 and 180 s, at which point animals were observed in their isolation cages for 10 min before being returned to

the colony room. Placenta donors and placenta-treated mice were no more closely related than second cousins.

Pain-Sensitivity Tests

Pain-sensitivity tests were performed between 10:00 and 12:00, 1 h after placenta or oil treatment, using a protocol modified from one employed by others in lab mice and rats (e.g., Vendruscolo et al., 2004, Weaver et al., 2007). An individual mouse was placed on a hot-plate set at 44.0 (± 1.0) ° C, and the latencies for mice to show nociceptive behaviors (see below) were measured. A pilot study indicated that this temperature was high enough to stimulate nociceptive behaviors without causing any tissue damage and was sensitive enough to detect inter-animal differences (unpub. data). Tests were performed in an environmental chamber (2.0 m x 1.3 m x 2.5 m) with temperature and humidity maintained at 23° C and 70%, respectively. Illumination was set to 1400 lux.

Ten to 20 minutes before each test, individual mice were moved in their isolation cages from the colony room to the environmental chamber and placed on the hot plate, which was contained by a plexiglass cylinder (6 cm height x 20 cm diameter). A ventilated plexiglass lid was placed over the cylinder to prevent the mice from standing upright and jumping out. The time from placement on the hot plate until shaking, licking or sustained lift of the hind paws, whichever occurred first, was recorded as an index of latency to nociception. Pilot data revealed that California mice frequently lick their front paws, so only hind-paw behaviors were used as measures of nociception (unpub. data). Mice were removed from the hot plate immediately after showing any of the above

behaviors. Animals that did not show any of these behaviors were removed from the hot plate after 120 s to prevent tissue damage. The hot plate was disinfected after each test. Mice were considered to have lower pain-sensitivity when they had longer latencies to show any nociceptive behaviors. Data from one placenta-treated virgin male, 3 placenta-treated expectants, and one oil-treated expectant were excluded from analysis because of problems with the hot-plate apparatus. The resulting sample sizes are shown in Table 1.

Exploratory and Caretaking Behavior Tests

Exploratory behavior and paternal motivation were determined using a modified open-field test (see below), beginning 4 h after placenta or oil administration. The open-field arena was a 1.0 m x 1.0 m square with a height of 0.80 m, constructed of opaque black plastic and placed on top of a clean sheet of white butcher paper to enhance contrast between the arena floor and the darkly colored mice. To prevent glare or reflection that might distract the subjects, the inner sides of the arena walls were sanded down. A digital camera was placed on top of the arena to record each test. After each test, the arena was disinfected and the butcher paper replaced. The open-field arena was located in an environmental chamber maintained at 1400 lux with two overhead white lights; temperature and humidity were maintained at 23°C and 70%, respectively. For each test, the male subject was initially placed in the center of the arena and video-recorded for 10 minutes, at which point a 1- to 4-day-old, unrelated pup was placed in the center of the arena for an additional 10 minutes (see below).

Exploratory behavior was quantified using TopScanLite software (Clever Sys Inc., Reston, Virginia, USA), which allowed us to track a mouse on a video and automatically measure several parameters of its movement. To quantify an animal's exploratory behavior the arena was divided into two distinct areas: an inner square, measuring 0.5 x 0.5 m, in the center of the arena, and an outer region extending 0.5 m from each wall to the perimeter of the inner square. Latency to cross the center of the arena, total distance moved, and duration spent in the inner square were determined for each mouse for the initial 10 minutes. Additionally, the number of times a mouse crossed between the inner and outer regions (i.e., bouts) was calculated. Mice with longer distances traveled, longer durations in the inner region, shorter latencies to cross the center of the open field, and/or higher number of bouts were considered to have higher motivation to explore the arena (Gould et al., 2009).

The behavioral response of mice to pups was quantified using JWatcher software (Blumstein & Daniel, 2007). For this 10-minute behavior test we measured latency to approach pups, latency to care for pups, and duration of caretaking behaviors (i.e., huddling + licking + carrying pup). One video of a placenta-treated virgin male was damaged and was not included in the analyses.

Statistical Analyses

Analyses were performed using R statistical software (R Core Team, 2014). Behavioral data were tested for normality using Shapiro-Wilk tests, and Bartlett's tests were used to determine homogeneity of variance. To determine any effects of placenta treatment or

reproductive condition on a male's pain sensitivity, exploratory behavior in the open field, or behavioral response to a pup, we compared measures using 2-way ANOVAs, with treatments (i.e., placenta or oil) and reproductive condition (i.e., virgins, expectants, fathers) as factors; post-hoc analyses were done using Tukey's HSD tests. For non-normally distributed data, we used separate Kruskal-Wallis tests using treatment or condition as factors. To determine if the behavior of mice during the open-field test was linked to their parental responsiveness we used Spearman's correlations between the total distances traveled by subjects during the initial 10-minute open-field test and the subject's paternal response (latency to approach and care for pups, time spent caring for pups). Correlational analyses for oil-treated and placenta-treated mice were performed separately.

Results

Pain Sensitivity

Latency to show nociceptive behaviors in the pain-sensitivity test did not differ between placenta- and oil-treated mice ($p=0.96$; Kruskal-Wallis test) or between fathers, first-time expectant males, and virgin males ($p=0.35$; Kruskal-Wallis test) (Table 3.1).

Exploratory Behavior

Placenta-treated males traveled longer distances in the open field than oil-treated males (main effect of treatment: $F_{2, 41}=8.90$, $p=0.004$; 2-way ANOVA), independent of

reproductive condition (main effect of reproductive condition: p=0.23, treatment x reproductive condition interaction: p=0.38; Figure 3.1). Neither placenta treatment nor reproductive condition affected males' latencies to cross the center of the open-field arena (effect of treatment: p=0.87, effect of reproductive condition: p=0.62; Kruskal-Wallis tests), duration spent in the inner region of the arena (effect of treatment: p=0.35, effect of reproductive condition: p=0.34; Kruskal-Wallis tests), or number of bouts (effect of treatment: p=0.54, effect of reproductive condition: p=0.20; Kruskal-Wallis tests) (Table 3.1).

Caretaking Behaviors

Neither treatment (placenta or oil) nor reproductive condition (new fathers, first-time expectants, virgins) affected latencies to approach pups (effect of treatment: p=0.20, effect of reproductive condition: p=0.20; Kruskal-Wallis tests). Moreover, oil-treated and placenta-treated mice did not differ in latency to care for pups (main effect of treatment: $F_{2, 40}=2.07$, p=0.15; 2-way ANOVA) or overall duration of caretaking behaviors (main effect of treatment: $F_{2, 40}=0.13$, p=0.71; 2-way ANOVA). On the other hand, we found a significant effect of reproductive condition on the latency to care for pups (main effect of reproductive condition: $F_{2, 40}=12.46$, $p<0.0001$, treatment x reproductive condition: p=0.26; 2-way ANOVA), in that fathers had shorter latencies to care for pups, compared to expectants (p=0.01) and virgins (p=0.004; Tukey's HSD test). Additionally, fathers showed longer durations of caretaking behaviors towards experimentally presented pups (main effect of reproductive condition: $F_{2, 40}=6.28$, p=0.001, treatment x reproductive

condition: p=0.90; 2-way ANOVA), when compared to expectants (p=0.04) and virgins (p=0.008; Tukey's HSD test) (Figure 3.2).

Correlations Between Exploratory and Caretaking Behaviors

Spearman's correlations from pooled data of all oil-treated mice revealed a negative relationship between the total distance traveled by a subject and its latency to approach a pup (ρ=-0.43, p=0.02, n=25). On the other hand, total distance traveled did not correlate significantly with either latency to care for a pup (ρ=-0.30, p=0.14, n=25) or duration of time spent caring for a pup (ρ=0.24, p=0.24, n=25).

In the case of placenta-treated mice, we found no significant correlations between the total distances moved by subjects and their latencies to approach (ρ=-0.34, p=0.10, n=23) or care for pups (ρ=-0.13 p=0.53, n=23), or the total time spent caring for pups (ρ=0.14, p=0.50, n=23).

Discussion

In this study, we investigated possible effects of placentophagia on pain sensitivity, anxiety-like behaviors, and parental care in male California mice. Additionally, we aimed to identify possible influences of reproductive condition (i.e., being a first-time father, first-time expectant father, or virgin male) on effects of placentophagia. We found that placenta treatment increased the exploratory behavior of male mice (i.e., total distance traveled during a 10-minute open-field test), independent of their reproductive condition.

Additionally, we found that our modified open-field paradigm, which we used to determine paternal motivation under presumably anxiogenic conditions, yielded differences in latencies to care for pups, as well as differences in caretaking behaviors, across reproductive conditions. Specifically, we found that first-time fathers had significantly shorter latencies to care for pups and longer durations spent caring for pups, when compared to expectant and virgin males. Finally, we found that the distances traveled by oil-treated males during the initial 10-minute open-field test were negatively correlated with their latencies to care for pups, suggesting a positive relationship between exploratory behavior and paternal motivation.

Studies on the causes and consequence of maternal placentophagia in mammals suggest that the main benefit of ingesting the afterbirth is to increase pain threshold (i.e., hypoalgesia) in mothers, due to changes in opioid signaling, as this may benefit both mothers and neonates (Kristal, 1991). This hypoalgesic effect has a rapid onset (~10 minutes) and is also found in male rats after placenta ingestion (Kristal, 1991). Contrary to our hypothesis, however, we did not find any effect of placenta ingestion on pain sensitivity of male California mice, as measured in a hot-plate test one hour after treatment with placenta. Moreover, we found no effect of reproductive condition on latencies for mice to show nociceptive behaviors. These findings suggest that neither placentophagia nor reproductive experience affects pain sensitivity in male California mice, at least in this test paradigm.

In a previous study, we found that adult, virgin male California mice administered a near-term placenta via oral gavage showed reduced latencies to approach a novel stimulus (i.e., neophobia), as well as reduced neural activity (i.e., Fos-immunoreactivity), in the dorsal area of the bed nucleus of the stria terminalis (BST), when compared to adult, virgin male mice administered oil vehicle (Perea-Rodriguez et al, in revision). These findings suggested that virgin males undergo behavioral and physiological changes after ingesting placenta that could be linked to changes in their state of anxiety, as neophobia is a specific component of anxiety and the BST is heavily involved in regulating anxiety (Walker & Davis, 1997). Results of the present study are consistent with this possibility: increased exploratory behavior in an open field, as seen in placenta-treated males, is typically interpreted as indicative of low anxiety. Together, therefore, our findings from these two studies suggest that placenta ingestion has anxiolytic effects in male California mice. On the other hand, neither study yielded evidence that placentophagia directly influences males' caretaking behavior toward experimentally presented pups.

Our finding of a negative correlation between distance traveled in the open field and latency to approach a pup in the same setting could be indicative of a negative relationship between anxiety-related behavior and paternal responsiveness. In other studies, we have similarly found negative correlations between anxiety-related behavioral or neural measures and indices of paternal responsiveness (Chauke et al., 2012, de Jong et al., 2012). On the other hand, several studies comparing behavioral and/or neural markers of anxiety between California mouse fathers and non-fathers have yielded mixed

results (Chauke et al., 2012, Hyer et al., 2016). Thus, while anxiety appears to be inversely related to males' responsiveness to pups, the effects of fatherhood on anxiety are not well understood.

In summary, this study is one of the first to investigate consequences of placentophagia in male mammals. The results are consistent with our previous findings that ingestion of placenta may have anxiolytic effects in males, thereby enhancing their willingness to approach pups, but that it may not directly influence their motivation to engage in caretaking behavior toward pups. Further studies should investigate the hormonal and neural mechanisms underlying effects of placentophagia on anxiety, as well as identifying the specific components of placenta that trigger these effects.

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Table 3.1: Behavior during a 10-minute open-field test of male California mice from different reproductive conditions (first-time fathers, first-time expectant fathers and virgin males) treated orally with either placenta in oil or oil alone. Data are presented as averages and standard errors. A total of 5 pain-sensitivity tests were unusable because of problems with the hot plate. Semicolons separate sample sizes for pain sensitivity and open-field tests.

Reproductive Condition	Treatment	Latency to Show Nociceptive Behaviors (s)	Number of Bouts (count)	Duration Inside Inner 50% of Arena (s)
First-Time Fathers	Oil n=8; 8	47.73 ± 16.99	23.75 ± 6.98	20.73 ± 6.83
	Placenta n=8; 8	42.77 ± 14.36	36.87 ± 6.77	37.68 ± 17.23
First-Time Expectant Fathers	Oil n=5; 8	15.21 ± 2.56	27.87 ± 28.17	24.66 ± 11.09
	Placenta n=7; 8	26.57 ± 7.71	43.25 ± 43.25	22.63 ± 8.12
Virgin Males	Oil n=9; 9	26.82 ± 6.55	39.88 ± 26.88	32.16 ± 7.46
	Placenta n=7; 8	17.91 ± 4.94	27.00 ± 5.85	15.97 ± 2.66

Figure 3.1: Total distance traveled (mean ± SE) by male California mice from different reproductive conditions (first-time fathers, first-time expectant fathers and virgin males) during a 10-minute open-field test treated orally with either placenta in oil (black bars) or oil alone (white bars). Total distance traveled was significantly higher in placenta-treated males than in oil-treated males, independent of reproductive condition.

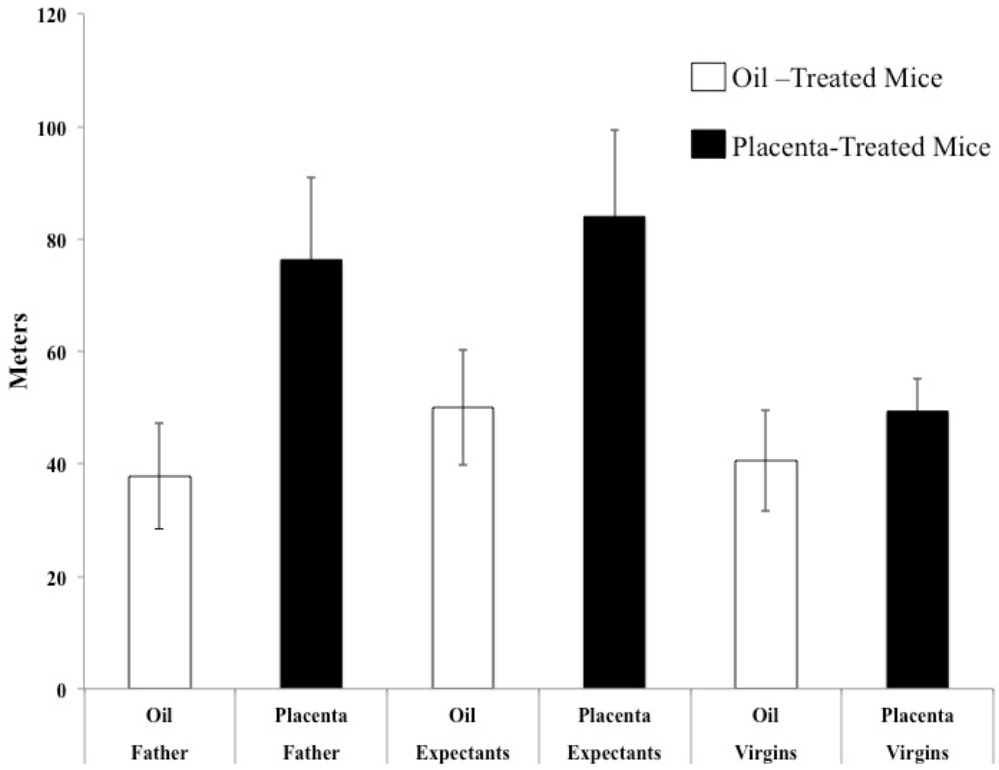


Figure 3.2: Behavioral responses to a pup presented in an open field for 10 minutes (mean ± SE), in male California mice from different reproductive conditions (first-time fathers, first-time expectant fathers and virgin males) treated orally with oil alone. Latencies to care for the pup and total durations spent caring for the pup were not influenced by treatment but differed among reproductive condition. Latencies to approach pups were not influenced by treatment or reproductive condition. See text for details.

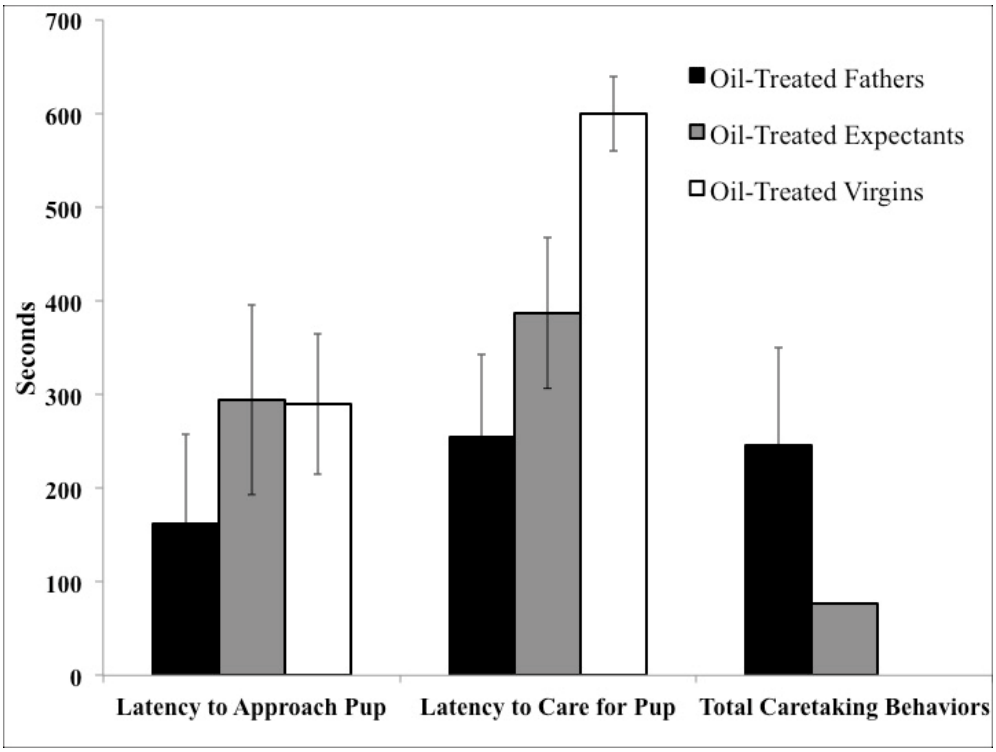
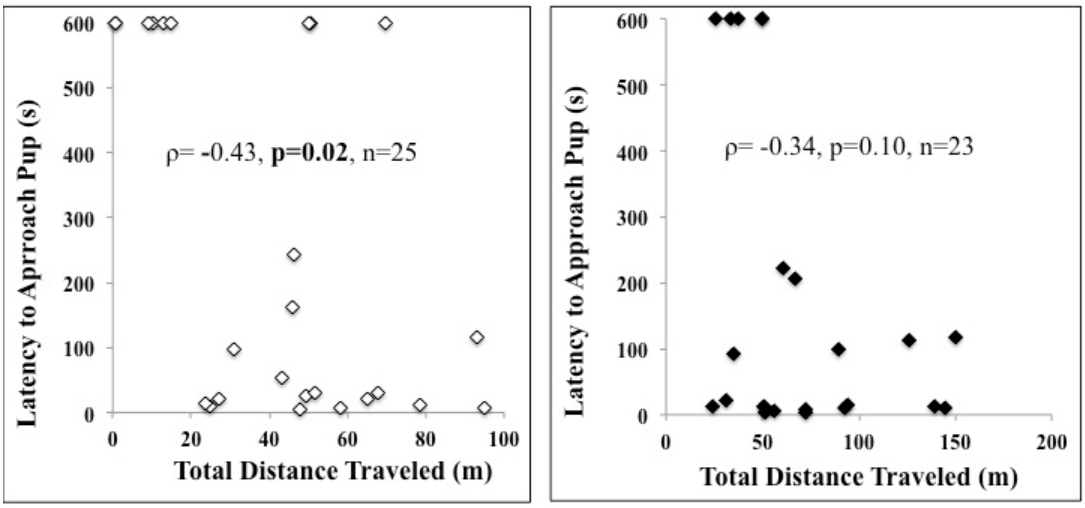


Figure 3.3: Spearman's correlations of total distances traveled and latencies to approach pups in an open field by oil-treated (left panel) and placenta-treated (right panel) adult, male California mice. Data are pooled across reproductive conditions (first-time fathers, first-time expectant fathers, and virgin males).



Chapter 4

Effects of Chemosensory Stimuli from Females on Paternal Behavior and Placentophagia in Adult, Virgin Male California Mice (*Peromyscus californicus*)

Juan P. Perea-Rodriguez [1, 2], Trynke R. de Jong [3], and Wendy Saltzman [1, 2]

[1] Department of Biology, [2] Evolution, Ecology, and Organismal Biology Graduate Program, University of California, Riverside, [3] Department of Behavioral and Molecular Neurobiology, University of Regensburg, Germany

Abstract

In California mice (*Peromyscus californicus*) and some other biparental rodents, sexually inexperienced (i.e., virgin) males vary in their attraction to placenta and to pups, but become highly attracted to pup-related stimuli when they gain reproductive experience. Chemical signals found in the excreta of lactating female California mice have been shown to be essential for the maintenance of fathers' parental responsiveness during the early post-partum period. Still unknown, however, is the possible role chemical signals from gestating females may have in the onset of caretaking behaviors and/or placentophagia. We hypothesized that chemosignals from pregnant females promote placenta ingestion and caretaking behavior in virgin male California mice. Thus, we continuously exposed adult virgin males to soiled bedding from either an unrelated pregnant female (n=14) or a virgin female (n=16), or to clean bedding (n=20). Males were placed in newly collected bedding every 2-3 days for 5-6 weeks (length of gestation period), after which their behavioral response to either conspecific placenta or an unfamiliar pup was measured. We also quantified vasopressin (AVP) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN), as AVP signaling in this region has been linked to the expression of caretaking behaviors in male California mice. Contrary to our hypothesis, continuous exposure of virgin male California mice to soiled bedding from either a pregnant female or a virgin female had no effect on their behavioral responses to placenta or pups and did not affect AVP mRNA expression in the PVN. It is likely that other factors important for reproduction in California mice (e.g.,

mating, cohabitating with a pregnant female, pair bonding) may play a more important or synergistic role in promoting caretaking behavior and placentophagia in males.

Keywords: California mice, placentophagia, caretaking behavior, paraventricular nucleus of the hypothalamus, vasopressin, in situ hybridization

Introduction

For some mammals, successful reproduction involves the care of offspring by both parents (i.e., biparental care). Interestingly, male parental care is present in fewer than 10% of all mammalian species, most of which are rodents, carnivores, and primates (Kleiman & Malcom, 1987). Most work on the onset and consequences of male parental care involves biparental rodents. Overall, these studies show that males undergo several behavioral, affective, and physiological changes with fatherhood, and that the presence of fathers significantly enhances the development and/or survival of their young (Saltzman & Ziegler, 2014, Bales & Saltzman, 2015). Thus, in biparental species, timely and appropriate expression of paternal behaviors is predicted to increase the parents' reproductive success (Trivers, 1974).

In some biparental rodent species, fathers show increased motivation to interact with pups and pup-related stimuli, compared to non-fathers (Brown, 1993). For example, California mouse (*Peromyscus californicus*) fathers show reduced latencies to approach and care for (foster) pups (Perea-Rodriguez et al., 2015), as well as longer periods of time spent in contact with pups, compared to age-matched, sexually inexperienced (i.e., virgin) males (de Jong et al., 2010). Male dwarf hamsters (Gregg & Wynne-Edwards, 2005) and California mice (Perea-Rodriguez & Saltzman, 2014) also become highly attracted to conspecific placenta with the birth of their young. Fathers in these species may assist their mates during parturition, helping pull neonates out as they are born, and ingesting placenta and amniotic fluid (i.e., placentophagia) in the process (Lee & Brown, 2002, Gregg & Wynne-Edwards, 2005). The functional significance of placentophagia in males

is unknown; however, we recently found that virgin male California mice treated orally with a near-term conspecific placenta showed reduced latencies to approach pups or a novel object (marble) 7 h after ingestion of placenta, compared to virgin males treated with oil vehicle (Perea-Rodriguez et al., in revision). In several mammalian species, placentophagia by mothers has been shown to enhance their maternal responsiveness, and for some species, ingestion of placenta is essential for mother-offspring recognition and bonding (Kristal, 1980).

In at least some biparental rodents, the expression of paternal care and placentophagia in fathers may be dependent on stimuli from their pregnant, parturient, and/or lactating mates. For example, in male prairie voles the maintenance of high levels of paternal behavior during the post-partum period, compared to the moderate levels of pup-directed care shown by non-fathers, is heavily influenced by the presence of their lactating mate (Jean-Baptiste et al., 2008). For male California mice, exposure to excreta from a lactating female is sufficient to maintain paternal responsiveness during the postpartum period, even in the absence of a female or pups (Gubernick, 1990). Importantly, characterization of the volatile component of excreta from breeding female California mice showed that excreta vary in their chemical composition throughout the gestation and lactation periods (Jemiolo et al., 1994).

Clearly, fathers in biparental rodent species can undergo changes in responsiveness to both pups and placenta with transitions between reproductive conditions. Still unclear, however, is the potential role that chemical stimuli from females, especially gestating females, may play in the onset of placentophagia and

paternal caretaking behaviors. In this study, therefore, we tested that hypothesis that olfactory stimuli from pregnant females facilitate the expression of caretaking behaviors and/or placentophagia, in adult, virgin male California mice. To do so we continuously exposed virgin males to bedding soiled by either a pregnant female or a virgin female, or to clean bedding, and evaluated their behavioral responses to unrelated pups and conspecific placenta. Virgin males are quite variable in their response to pups (Chauke et al., 2012, de Jong et al., 2012) and are not usually attracted to placenta (Perea-Rodriguez & Saltzman, 2014). To determine if exposure to chemical cues from pregnant females also facilitates neuroendocrine changes in males that might promote parental care, we quantified mRNA expression for vasopressin (AVP) in the paraventricular nucleus of the hypothalamus (PVN). AVP is important for paternal care, stress, aggression, and anxiety (Numan & Insel, 2003). We previously found that AVP mRNA levels in the PVN of adult virgin males were positively correlated with their latencies to approach and investigate an unrelated pup (de Jong et al., 2009), suggesting a negative relationship between AVP mRNA levels in the PVN and parental motivation. We predicted that males exposed to bedding from a pregnant female would show increased attraction to both pups and placenta, compared to males exposed to either bedding from a virgin female or clean bedding. Additionally, we predicted that males exposed to excreta from a pregnant female would have lower AVP mRNA expression in the PVN than males exposed to excreta from an unrelated virgin female or to clean bedding.

Methods

Animals

We used California mice that were born and reared in our breeding colony at the University of California, Riverside and that were descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA) (de Jong et al., 2013). Mice were housed in shoebox-style, polycarbonate cages (44 × 24 × 20 cm) containing aspen shavings for bedding (~40 grams) and cotton wool (~5 grams) for nesting material, with *ad libitum* access to food (Purina Rodent Chow 5001, PMI Nutrition International, St Louis, MO, USA) and water. Lights were on a 14:10 light:dark cycle, with lights on from 05:00 to 19:00 h. Ambient temperature was approximately 23°C, and humidity was approximately 65% (Saltzman et al., 2015). Animals were checked daily and cages were changed weekly.

At 27–32 days of age, prior to the birth of younger siblings, juveniles were removed from their natal cages and housed in same-sex groups containing 3-4 age-matched, related and/or unrelated mice. When mice reached the age of sexual maturity (~90 days of age: Gubernick & Alberts, 1987), they were either placed into breeding pairs or housed in virgin male or virgin female pairs with a cagemate from their original same-sex group (see below). Pair-housed mice were no more closely related to each other than first cousins.

Experimental Design

Virgin male pairs were randomly assigned to one of three experimental groups, in which they were continually exposed to: (1) soiled bedding from an unrelated, pregnant female (PF males; n=14), (2) soiled bedding from an unrelated, virgin female (VF males; n=16), or (3) clean bedding (CB males; n=20). Each PF or VF male was exposed to soiled bedding from a single pregnant female throughout the gestation period (Gubernick 1988), or a single virgin female, respectively. After 5-6 weeks of exposure, each PF, VF, and CB virgin male was tested with one of two stimuli: a near-term, conspecific placenta from an unrelated pregnant female (n=7-10 males per group) or a 1- to 4-day-old unrelated pup (n=7-10 mice per group). Within each virgin male pair, the two males were exposed to the same bedding stimulus but were tested with different stimuli (pup or placenta).

At the time that the virgin male pairs were formed, 16 breeding pairs were also formed Fathers from these pairs were tested with an unfamiliar pup or placenta from an unfamiliar female (n=8 per stimulus) 1-4 days after the birth of their first litter of pups. All mice were weighed twice per week throughout the experiment.

Collection of and Exposure to Bedding

To estimate the day of copulation in the bedding/placenta donors and the breeding pairs, prior to pairing we housed each male and female on opposite sides of a cage divided into two compartments with a removable, perforated Plexiglas divider, through which the mice could smell, hear and see, but not touch, each other. Each compartment measured approximately 22 x 12 x 10 cm and provided access to food, water, and nesting material. After one week the divider was removed, mice were observed for 2 hours, and

copulations were noted. Approximately 75% of the pairs mated within 2 hours after pairing. Mating is rarely seen within this time window when mice are paired without this familiarization process (unpub. obs). The remaining pairs that were not seen mating were left undisturbed for 2 days. Monitoring the presence of sperm plugs in females is not a reliable method for determining the timing of copulation in this species, as males and females will sometimes eat the sperm plug (pers. obs), and sperm plugs are detected in fewer than half of newly mated females (Gubernick, 1994).

Virgin male pairs in each group were exposed continuously to their assigned bedding type (from a pregnant female, from a virgin female, or clean). Bedding was changed every 2-3 days. For donor pairs that were seen copulating immediately after pair formation, collection of bedding from each pregnant female began one day later. For donor pairs that were not seen copulating at pairing, bedding collection from the female began 48 hours after pair formation. In order to collect soiled bedding from pregnant females while avoiding bedding from their mates, the night before each bedding collection bedding-donor pairs were placed in a clean cage in which the male and female were separated by a removable divider as described above. The male and female of each donor pair remained in separate compartments overnight (17:00-09:00 h), and were reunited in a clean cage the following morning. All of the soiled bedding and cotton from the compartment that had housed the pregnant female was placed into a new cage with ~30 grams of clean bedding, food and water, and the virgin male pair assigned to that specific female donor was transferred to it. This ensured that virgin males in the PF condition were exposed to the excreta of pregnant females throughout the complete

pregnancy. Identical procedures were performed for collection and exposure of bedding from virgin females. In the case of Fathers and CB males, pairs were placed in a new cage with fresh bedding, cotton, food, and water, on the same schedule as cage changes in the PF and VF groups.

Behavior Tests and Tissue Collection

To control for the length of time males remained housed in either breeding or virgin male pairs, all mice were tested 1-4 days after breeding pairs gave birth to their first litter of pups. At this time, an unrelated, near-term pregnant female (different from the assigned bedding donor) was euthanized and her placentas were harvested as described previously (Perea-Rodriguez & Saltzman, 2014). Placentas were placed in a small plastic weighing boat (2.5 cm diameter x 0.95 cm deep) containing 0.5 mL of saline before being used for behavioral testing 60-90 minutes later (see below).

Before being tested, each mouse was isolated for 30-90 minutes in a clean cage with bedding, food and water. At the beginning of the test, either an unrelated, 1- to 4-day-old pup or a placenta from an unrelated female was placed at the opposite end of the cage from the focal male, and the male was video recorded for 10 minutes. Immediately after the conclusion of the behavioral test the focal male was decapitated and the brain snap-frozen for histological analyses (see below). Videos were later scored by a single observer using JWatcher software (Blumstein & Daniel, 2007). For males tested with a pup, we scored the total duration of caretaking behavior (i.e., huddling pup, grooming pup), latency to approach pup, and latency to show caretaking behaviors. For males tested with a placenta we scored latency to approach and ingestion of placenta. Mice

were considered placentophagous if they ate some or all of the experimentally presented placenta. Mice were tested during the lights-on phase of the light:dark cycle, between 10:00 and 14:00 h.

In Situ Hybridization

We quantified gene expression as previously described (de Jong et al., 2012). Frozen brains were sliced on a cryostat into six series, each made up of twenty 20 um-thick sections, which included the PVN (Figure 4.1). Sections were thaw mounted onto gelatin/chrome-alum coated glass slides. One series was air-dried and stained with Quick Stain (American MasterTech, Lodi, CA) to determine the location of the PVN. A total of 11 brains were damaged by the collection process and another 10 were damaged during the slicing procedure; these were unusable for analyses. Final sample sizes for each group are shown Figure 4.2.

Levels of AVP mRNA were quantified using a ^{35}S-labeled deoxyoligonucleotide probe (Sigma Genosys, Woodland, TX). The probes were complementary to the 3' end of the glycoprotein sequence for rat (*Rattus norvergicus*) AVP gene (48-bp oligomer: GTAGACCCGGGGCTTGGCAGAATCCACGGACTCTTGTGTCCCAGCCAG). Frozen sections were fixed in freshly made 4% buffered paraformaldehyde for 20 min, followed by dehydration and rehydration through graded ethanol solutions. Sections were exposed to 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8) for 8 min and were dehydrated through graded ethanol solutions. Sections were hybridized overnight (20 h) in a humidified chamber at 42° C with 0.20 x 10^6 CPM of labeled probe dissolved in a buffer solution (50% formamide, 5X SET, 0.2% SDS, 5X Denhart's, 0.5 mg/mL salmon

sperm DNA, 0.25 mg/mL yeast tRNA, 100 mM dithiothreitol, and 10% dextran sulfate; 30 mL per section). After hybridization, sections underwent serial washes in saline sodium citrate (SSC): 4X SSC for 5 min at room temperature, 2X SSC for two times 30 min at 55° C, and 1X SSC and 0.3X SSC for 30 min each at room temperature. Sections were then dehydrated through graded ethanol solutions containing 0.3 M ammonium acetate followed by 95% and 100% ethanol and air-dried.

Sections were placed in autoradiography cassettes and apposed to film (Kodak BioMax MR Film, Eastman Kodak, NY). Sections containing the PVN (2-5 per animal) were spread over two cassettes, and films were developed after 4 days. Developed films were digitized and analyzed using the ImageJ software program from the National Institutes of Health. Gray levels of the ^{14}C-standard on each film were measured and fitted to a curve expressed in nCi/g, and hybridization and background signals on the same film were quantified using that curve. Each positive signal in the PVN was outlined twice, and a neutral area immediately adjacent to the PVN was outlined twice in order to obtain reliable measurements of signal and background optical densities (Figure 4.1). The final densities were calculated by subtracting the average background densities from average signal densities.

Statistical Analysis

Analyses were completed using R statistical software (R Core Team, 2014). Fisher Exact-Bachloo's tests were used to determine if the experimental groups (PF, VF, CB, fathers) differed in the proportion of mice that cared for pups or ingested placenta. Behavioral and neuroendocrine data were tested for normality using Shapiro-Wilk tests, and Bartlett's

tests were used to determine homogeneity of variance. Because behavioral and mRNA data were not normally distributed they were analyzed using Kruskal-Wallis tests with experimental group as a factor. We performed Spearman's correlations using pooled data from all virgin male groups to determine if their behavioral responses to placentas (latency to approach placentas) or pups correlated with AVP mRNA expression in the PVN.

Results

Behavioral Responses to Placenta

Overall, 18 out of 32 males (56%) ate some or all of the experimentally presented placenta. The proportion of males that engaged in placentophagia did not differ significantly among experimental groups (all pairwise comparisons: $p \geq 0.12$; Fisher Exact-Bachloo's tests). Additionally, latencies to approach placentas did not differ among groups ($p=0.91$; Kruskal-Wallis test; Table 4.1).

Behavioral Responses to Pups

Twenty-five out of 31 males (78%) showed caretaking behaviors (huddling and/or grooming) towards experimentally presented pups. The proportion of males that engaged in caretaking behavior did not differ significantly among conditions (all pairwise comparisons: $p \geq 0.08$; Fisher Exact-Bachloo's tests). Additionally, males' quantitative behavioral responses to pups did not differ among experimental groups ($p=0.35$; Kruskal-Wallis test; Table 4.1).

AVP mRNA Expression

Positive hybridization signals were found for AVP mRNA in the PVN, but AVP mRNA expression did not differ among experimental groups ($p=0.89$; Kruskal-Wallis test; Figure 4.2). The average signal intensity was 2265.44 nCi/g ($n=41$), which is higher than what has been reported previously for adult, virgin male California mice (1947.11 ± 162.87 nCi/g: de Jong et al., 2012) and somewhat lower than for female rats using the same hybridization protocol (~2600 nCi/g: Ivy et al., 2008).

Correlations Between Behavior and AVP mRNA Expression

Spearman's correlations did not reveal any significant associations between AVP mRNA expression in the PVN and males' behavioral responses to pups or placenta (Table 4.2).

Discussion

The goal of this study was to determine the possible role chemical cues from gestating females (found in excreta) may play in modulating the onset of placentophagia and/or caretaking behavior in adult, virgin male California mice. Virgin males of this species are highly variable in their paternal responsiveness (de Jong et al., 2012, Chauke et al., 2012), whereas fathers, both new and experienced, consistently exhibit caretaking behaviors toward their own or unfamiliar pups (de Jong et al., 2013, Perea-Rodriguez et al., 2015). Similarly, virgin male California mice are not likely to eat experimentally presented conspecific placenta, but males become attracted to placenta with the onset of their mate's pregnancy (Perea-Rodriguez & Saltzman, 2014). Together, these findings suggest that mating, pair bonding and/or cohabitation with a pregnant female may

influence a male's response to both placenta and pups. We hypothesized, therefore, that continuous exposure of virgin males to excreta from a pregnant female throughout her complete pregnancy would influence their behavioral responses to placenta and pups. Contrary to our predictions, we found no evidence that exposure to soiled bedding from an unrelated, unfamiliar pregnant (or virgin female) increased virgin males' attraction to placenta or pups, compared with exposure to clean bedding. Similarly, we found that vasopressin (AVP) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) did not differ among mice from the different experimental groups.

Previous work on the California mouse has shown that the volatile components of excreta from a lactating, conspecific female (whether pair bonded to the subject or not) are sufficient to maintain paternal behaviors during the early post-partum period, in the absence of any other stimuli from females and/or pups (Gubernick et al., 1994). We found that these chemical stimuli from females may not be enough to facilitate placentophagia or parental care in adult, virgin males. Our results suggest that other factors important for reproduction in the California mouse (e.g., mating, pair bonding, cohabitation with a female) may have a more important role in promoting attraction to pups and placenta than exposure to chemical cues from females. For instance, California mice mate soon after the birth of their pups, as females of this species experience post-partum estrus (Gubernick, 1988). For rodents, mating results in direct changes in endocrine and/or neurotransmitter signaling (e.g., oxytocin) that can influence how individuals respond to social stimuli, such as stimuli from their mates or young (Numan & Insel, 2003). For some biparental rodents, these neuroendocrine changes are important

for social attachment (Numan & Insel, 2003). It is possible that these neuroendocrine effects of mating might prime males to respond to (chemosensory) stimuli from their mates. Consistent with this possibility, male California mice are more likely to ingest placenta with the onset of their mate's first pregnancy than prior to being paired with a female (Perea-Rodriguez & Saltzman, 2014).

Changes in intracerebral AVP signaling have been linked to males' parental responses in several biparental rodents, including the California mouse (Bales & Saltzman, 2015). In this species, AVP mRNA levels in both the bed nucleus of the stria terminalis (BST) (Bester-Meredith & Marler, 2003) and the PVN (de Jong et al., 2012) correlate negatively with paternal motivation. Additionally, California mouse fathers have reduced mRNA expression of AVP V1a receptors in the BST compared to virgin males (Perea-Rodriguez et al., 2015), although we previously found that AVP mRNA expression in the PVN (the main source of AVP) is not affected by fatherhood (de Jong et al., 2013). This was corroborated by our present findings, as AVP mRNA expression in the PVN did not differ between first-time fathers and virgins, whether exposed to female excreta or not. It is possible, therefore, that exposure to excreta from a pregnant female might affect AVP signaling in the brain, but through changes in receptor expression rather than in AVP synthesis or secretion, Alternatively, mating or cohabitation with an opposite-sex cage mate, instead of chemosignals alone, could influence caretaking behavior and AVP signaling, as demonstrated in prairie voles (Bamshad et al., 1994).

Some caveats should be taken into consideration when interpreting the results of this study. First, it is possible that the exposure regimen in our experiment did not

adequately mimic exposure to female chemical cues that pair-bonded males typically experience. Second, the procedure itself (i.e., bedding/cage changes), independent of the specific conditions, could have affected the mice's behavior and AVP expression, possibly by stressing the animals. Third, chemosignals from females might play a role in activating care-taking behavior and placentophagia in males, but only with synergistic effects of other reproductive experiences or cues (e.g., mating or cohabitation with a female).

In summary, this study indicated that adult, virgin male California mice do not undergo changes in their behavioral responses to placenta and/or pups, or in their expression of AVP mRNA in the PVN, after being exposed to excreta from an unfamiliar pregnant or virgin female. Further work should investigate possible effects of mating, cohabitation, and/or pair bonding on the activation of placentophagia and caretaking behaviors in new fathers.

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Table 4.1: Behavioral responses of virgin males and first-time fathers to placentas or pups, as well as AVP mRNA expression in the PVN. Data shown are either numbers and percent of animals performing the behavior (placenta ingestion, caretaking behavior) or medians (first and third quartiles in parentheses) (latencies and durations of behaviors).

Response to placenta		Response to pup					
Number that ate placenta	Latency to approach (s)	Number that exhibited caretaking behavior	Latency to approach (s)	Latency to exhibit caretaking behavior (s)	Investigate (duration) (s)	Caretaking behavior (duration) (s)	AVP mRNA in PVN (nCi/g)
6 of 8 (75.0%)	62.22 (34.63, 65.57)	7 of 7 (100%)	60.19 (12.83, 110.76)	108.12 (64.29, 307.62)	94.74 (31.2, 154.35)	869.68 (716.82, 1017.75)	2468.49 (1912.72, 2664.52)
4 of 6 (66.7%)	48.14 (18.98, 466.99)	5 of 7 (71.4%)	39.68 (73.43, 85.76	238.97 (109.57, 848.06)	37.43 (34.98, 54.75)	844.99 (351.93, 1061.60)	2643.46 (1623.53, 2905.59)
4 of 8 (50.0%)	61.63 (43.54, 600)	6 of 7 (85.7%)	20.72 (9.20, 96.66)	224.13 (165.54, 649.89	104.28 (77.30, 134.81)	794.08 (446.08, 997.86)	2397.78 (2225.50, 2863.97)
4 of 9 (44.4%)	209.10 (24.94, 320.95)	7 of 10 (70.0%)	122.32 (66.82, 226.83)	164.17 (116.17, 954.31)	33.55 (14.10, 78.94)	1008.62 (244.65, 1039.13)	2044.36 (2044.36, 1919.54)

Table 4.2: Spearman's correlations of behavioral responses of adult, virgin California mice to a near-term, conspecific placenta, or an unrelated pup, and vasopressin (AVP) mRNA expression in the paraventricular nucleus of the hypothalamus (PVN).

	Latency to Approach Placenta (s)	Latency to Approach Pup (s)	Latency to Care for Pup (s)	Total Caretaking Behaviors (duration)	Investigate Pup (duration)
AVP mRNA Expression in the PVN	ρ=0.29 p=0.28 n=23	ρ=-0.05 p=0.84 n=24	ρ=0.12 p=0.65 n=24	ρ=-0.09 p=0.74 n=24	ρ=-0.38 p=0.15 n=24

Figure 4.1: Diagram of the paraventricular nucleus of the hypothalamus in *Mus sp.* (circle). Taken from the Mouse Brain Library (www.MLB.org).

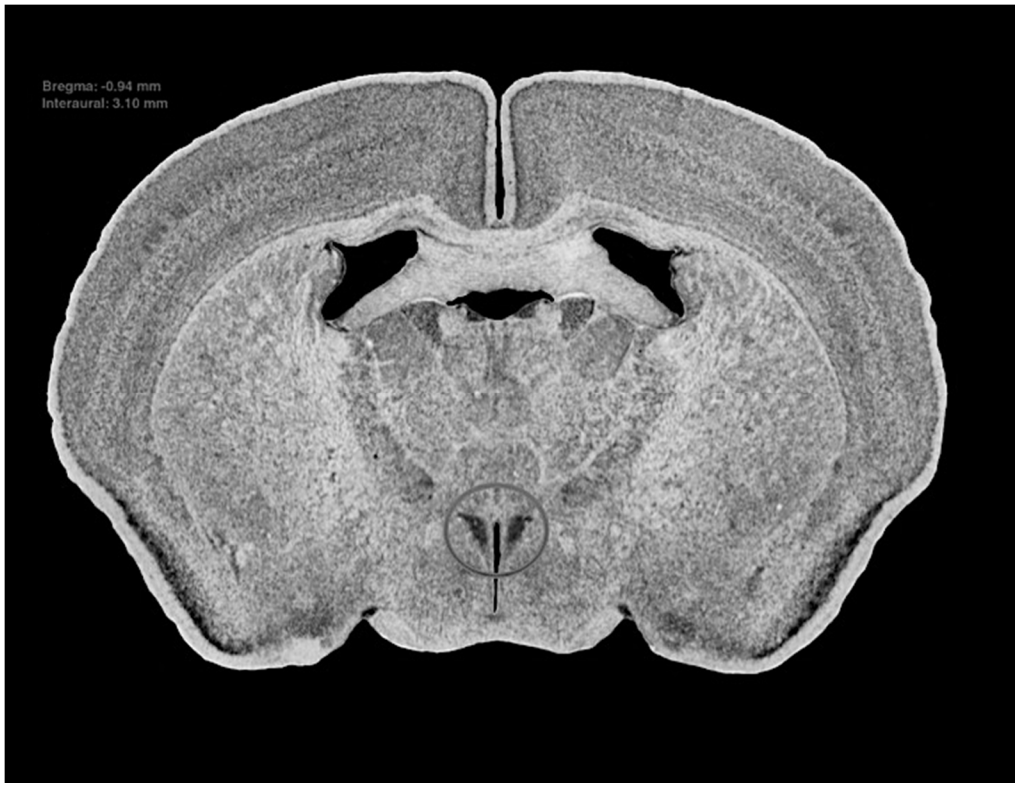
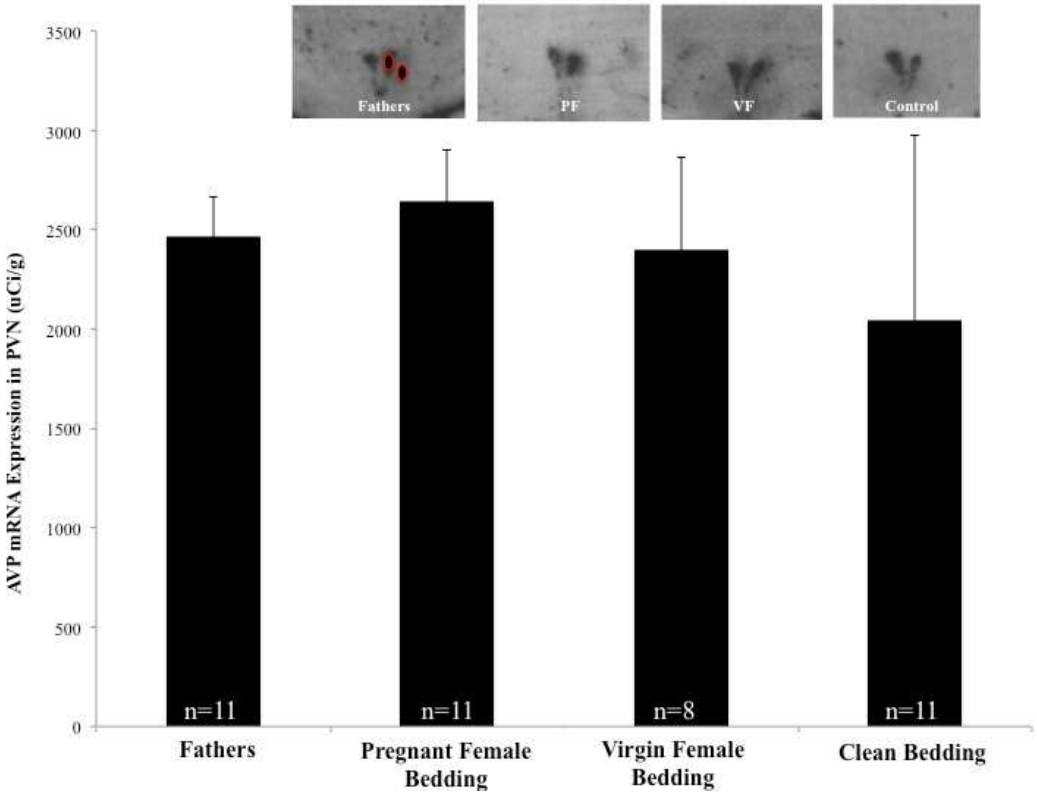


Figure 4.2: Vasopressin (AVP) mRNA expression in the paraventricular nucleus of the hypothalamus of male California mice exposed to placenta and pups (pooled). Data shown are medians and first and third quartiles; samples sizes are shown within each bar.



DISSERTATION CONCLUSION

My dissertation addressed the causes and consequences of placentophagia in the California mouse, specifically as it relates to paternal care. Aim 1 investigated the possible role reproductive experience may have in placentophagia in males and females, whereas Aim 4 sought to determine if chemosignals from gestating females may promote attraction to placenta and/or pups in males. Aims 2 and 3 of my dissertation investigated the possible changes in neophobia, exploratory behavior, pain sensitivity and/or paternal behaviors males undergo when they ingest placenta. Based on the available literature on maternal placentophagia, I hypothesized that males would become attracted to placenta and pups as they gained reproductive experience, and predicted that the process that leads to this behavioral change is mediated by chemical signals found in the excreta of pregnant females. Furthermore, I hypothesized that placenta ingestion by adult males would lead to behavioral changes. Specifically, I predicted that male placentophagia would lead to decreased pain sensitivity and anxiety-like behaviors (i.e., neophobia, increase exploratory behaviors), which would result in an enhanced paternal response.

Overall, the data from this dissertation suggest that males and females become attracted to placenta when they gain reproductive experience (i.e., parturitional/parental experience for females, and sexual, parental and/or birthing experience for males: Aim 1), and that placenta ingestion by males can lead to behavioral changes as soon as 4 hours post-ingestion (Aim 3), but not 24 h post-ingestion (Aim 2). Specifically, placenta-treated male mice showed shorter latencies to approach novel stimuli (i.e., pups, or pup-sized marbles) 7 h post-treatment (Aim 2), as well as longer distances traveled in an open field

4 h post-treatment (Aim 3) compared to oil-treated controls. Contrary to my prediction, placenta treated males did not differ in their behavioral response to pups when compared to oil-treated mice (Aims 2 & 3), but total distances traveled by oil-treated subjects, independent of reproductive condition, were positively correlated with their paternal motivation (i.e., negatively correlated to their latencies to approach pups: Aim 3). Finally, Aim 4 of my dissertation revealed that continuous exposure of adult, virgin-male California mice to soiled bedding from a pregnant was not enough to affect their behavioral response to placentas or pups. Taken together these findings suggest that males become attracted to the afterbirth when their mates become pregnant, and that ingesting placenta can modify a male's response to averse/novel stimuli. Additionally, these results suggest that the chemical signals of gestating females do not influence placentophagia or paternal care, and that possibly other important events in the reproduction of this species (e.g., mating) may have a more dominant effect on responses to placentas and pups by adult, virgin males.

The available data on the consequences of placentophagia by mothers suggests that ingesting placenta can result in physiological changes in mothers that can prolong the investment in their young. Similar to the available data on placentophagia by females, the data from this dissertation suggests that placentophagia is not having any detectable effect on direct parental care. In females, it seems to facilitate parturition, and possibly, feeding of neonates through changes in pain sensitivity in new mothers, as newborn may attach more easily to nipples if mothers have a higher pain threshold. In male California mice, placentophagia is not having any effect on direct parental care. One possibility is

that placentophagia by males may be having an effect on indirect paternal care through changes in exploratory behaviors. From the resulting data from my dissertation, it is difficult to interpret the link between changes in neophobia and exploratory behavior and paternal care, but neonates may benefit if fathers are defending their territories or gathering food for themselves and/or their mates. Further work on the possible relationships between paternal responsiveness and anxiety-like behaviors could give insight into the possible function/benefit of placentophagia to parents and/or offspring of changes in exploratory behaviors in new fathers.

An important finding of this research is that there was a significant influence of reproductive experience on the behavioral responses of male mice to pups in an anxiogenic environment. Results from our modified open-field tests showed that first-time fathers are more parentally responsive than first-time expectant males and virgin males under this paradigm. These findings suggest that anxiety has an important impact on a male's paternal response, and that males may undergo changes in their responses to aversive stimuli when they reproduce.

The results from this dissertation provide novel information regarding the possible factors influencing the onset of placentophagia by males of a biparental mammal, as well as the possible behavioral changes males undergo when they ingest placenta. To date this is the only study that has studied the consequences of this behavior in a biparental species, which provides insight into the possible effect of placentophagia in influencing parental care in this species.