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Behavioral and endocrine consequences of placentophagia in male California mice (*Peromyscus californicus*)

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ABSTRACT

Ingestion of placenta by mammalian mothers can lead to changes in pain sensitivity, hormone levels, and behavioral responses to newborns. In some biparental mammals, males, in addition to females, ingest placenta when their offspring are born. In the monogamous, biparental California mouse (*Peromyscus californicus*), males first become attracted to placenta when cohabitating with their pregnant mate, and virgin males administered placenta are less neophobic than males given oil vehicle. In this study, we investigated the effects of placentophagia on pain sensitivity, anxiety-like behavior, behavioral responses to pups, and circulating corticosterone levels of both breeding and nonbreeding male California mice. We orally administered either a conspecific placenta or oil vehicle to male mice from three reproductive conditions (first-time fathers, first-time expectant fathers, and virgin males) and tested their pain sensitivity 1 h later, as well as their exploratory behavior and paternal responsiveness in an open field 4 h post-treatment. We measured plasma corticosterone immediately after the open-field test. We found that placenta-treated males, independent of reproductive condition, traveled significantly longer distances in the open field than males treated with oil, indicative of lower anxiety. Additionally, fathers had shorter latencies to approach and to care for pups (i.e., huddling and licking pups), and spent more time engaging in these behaviors, than did age-matched expectant fathers and virgin males, independent of treatment. We found no effect on plasma corticosterone levels or pain sensitivity as a result of either treatment or reproductive condition. These findings indicate that placenta ingestion decreases anxiety-related behaviors in male California mice, but might not influence pain sensitivity, paternal responsiveness, or plasma corticosterone concentrations.

1. Introduction

Female mammals typically consume placenta after giving birth, with some exceptions (e.g., marine mammals, humans, and camelids: [19]). The functional significance of this behavior is unclear, but proposed explanations include (a) general nutrition (i.e., many parturient females become aphagic before labor and are motivated to eat the highly nutritional placenta during or after parturition), (b) specific nutrition (i.e., mothers lack a specific hormone or biologically active factor that is found in placenta, and replenish it through placentophagia), and (c) predator and pathogen avoidance [18]. Regardless of the ultimate explanation for placentophagia, some female mammals change their behavioral response to placenta across changes in their reproductive condition. Specifically, females' response to placenta changes from aversion when they are sexually inexperienced to attraction during late pregnancy or with birthing experience [18,20]. This behavioral transition has been reported in rats (*Rattus norvegicus*; [18]), house mice (*Mus musculus*; [18]), California mice (*Peromyscus californicus*; [27]), Siberian hamsters (*Phodopus sungorus*; [9]), Djungarian hamsters (*P. campbelli*; [8,9]), rabbits (*Oryctolagus cuniculus L.*; [25]), and sheep (*Ovis aries*) [23].

After ingesting placenta, mothers may undergo specific physiological changes that can potentially affect maternal responsiveness and opioid signaling. For example, placentophagia increases opioid-mediated analgesia in female rats [1]. In addition to pain sensitivity, this effect on the opioid system may influence maternal behavior, as ingestion of placenta and amniotic fluid by adult sexually naïve female rats enhances the stimulatory effect of intracerebroventricular morphine treatment on maternal sensitization (i.e., pup-induced maternal responsiveness: [26]). A recent double-blind study in humans revealed that women who ingested dehydrated encapsulated placenta showed a small but significant differences in protein and steroid hormonal profiles of women taking placenta capsules compared with women who ingested control capsules containing beef (Young et al. [33]). These

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findings suggest that placentophagia may result in distinct physiological changes in female mammals, which in turn may positively influence several aspects of maternal care.

In several biparental mammals (i.e., both males and females provide care for their offspring), males, as well as females, ingest placenta during the birth of their infants. In primates, for instance, placentophagia by males has been seen 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.). Among biparental rodents, male placentophagia has been reported in Djungarian hamsters [17], California mice [22], and prairie voles (*Microtus ochrogaster*; K.L Bales, pers. comm.). 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 [9]. In uniparental rats, males are more likely to ingest placenta after increased exposure to the afterbirth [19]. Moreover, studies in the Djungarian 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 significantly less likely to ingest placenta when sexually inexperienced than when their mate is pregnant [9,27].

Only two studies have investigated the physiological and 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 neural and behavioral changes after ingesting placenta ([1]; Perea-Rodriguez, unpub. Ph.D. dissertation). Adult male rats, similar to adult females, experience an increase in opioid-mediated analgesia, suggesting that placentophagia may modify opioid signaling pathways [1]. Adult virgin male California mice administered placenta homogenized in sesame oil via oral gavage showed lower latencies to approach novel stimuli (i.e., an unrelated pup or a pup-sized-marble), than mice administered oil vehicle only. Additionally, oral administration of placenta to adult virgin male California mice resulted in decreased expression of Fos (a marker for neuronal activity: [14]) in the dorsal bed nucleus of the stria terminalis for up to 4 h post-administration. The bed nucleus of the stria terminalis is a brain area involved in regulating fear and anxiety in rodents (i.e., [31]) (Perea-Rodriguez, unpublished Ph.D. dissertation). However, no changes in caretaking behaviors were seen as a result of placenta treatment of virgin male mice (Perea-Rodriguez, unpublished Ph.D. dissertation). These data indicate that placentophagia may reduce anxiety-related and stress-related responses to novelty. Though the mechanisms of these effects are unknown, it may very well be through the vagus nerve as has been identified for the opioid-mediated analgesic effect of placentophagia in female rats (Tarapacki et al. [29]). Alternatively, any hormonally mediated effects of placentophagia could be related to steroid hormones, as these hormones readily cross the blood-brain barrier and are biologically active following ingestion.

One possible ultimate explanation for the presence of male placentophagia in monogamous biparental mammals is that this behavior induces physiological changes in males that may influence how new fathers behave when they first encounter their pups, and thus may be one of the factors regulating the onset of paternal care [27]. Recent work on California mice suggests that fathers may be less anxious than non-fathers [16], and that anxiety-related neural and behavioral measures correlate negatively with certain measures of paternal responsiveness [5,6,21]. Additionally, male California mice undergo changes in pain sensitivity during the pair-bonding process [34], possibly mediated by the changes in hypothalamic-pituitary-adrenal (HPA) axis activity they experience when they pair bond [2,5,24]. Thus, it is possible that placentophagia might further impact pain sensitivity, fear and anxiety-like responses, and parental responsiveness in males and facilitate interactions between fathers and their young.

In this study, we evaluated the possible behavioral and physiological effects of placenta ingestion in male California mice. We hypothesized that placenta ingestion leads to changes in HPA activity, and predicted that administration of placenta, vs. oil vehicle, would lead to reduced pain sensitivity, lower corticosterone responses to experimental manipulations, decreased neophobia, and increased paternal responsiveness. To test this hypothesis, we characterized pain sensitivity, exploratory behaviors in an open field, behavioral responses to an unrelated pup, and corticosterone responses to our test paradigm in adult male California mice that were treated orally with either a conspecific placenta or oil vehicle. Because other reproduction-related stimuli from mating or cohabitation with a pregnant female might influence males' responses to placentophagia, we compared these effects in virgin males, males whose mates were pregnant with their first litter, and first-time fathers.

2. Methods

2.1. Animals

We used male California mice born and reared in our colony at the University of California, Riverside, that descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were housed in standard, shoebox-style polycarbonate cages (44 × 24 × 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 maintained at approximately 23 °C and 70%, respectively. Mice were checked twice daily, and cages were changed weekly.

We removed mice from their parents' cage at 27–31 days of age, before the birth of their younger siblings, and housed them in same-sex groups of three or four age-matched individuals. These groups contained no more than two siblings from any one litter. As they reached the age of sexual maturity (~90 days: [10]), males and females were paired with either an unrelated same-sex mouse from their original virgin group or an unrelated opposite-sex adult (see below).

2.2. Experimental design

We randomly assigned male California mice to one of the following reproductive conditions: sexually inexperienced males (i.e., virgins), first-time expectant fathers, and first-time fathers. Virgins (n = 16) were paired with an unrelated male 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 virgin female. Male and female pair mates were no more closely related than second cousins. We weighed all mice twice weekly throughout the study and monitored pregnancies by body-mass changes in females.

Two to 5 days after the birth of pups to a breeding pair, the father from that pair 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 g) from an unrelated conspecific female, homogenized in sesame oil via oral gavage (see below), whereas the remaining half of the males in each reproductive condition were administered oil alone (controls). Rodents lack the emetic reflex and are unable to vomit, which facilitates the gavage procedure and placenta and oil administration [15]. Sesame oil was used because it allowed for the complete homogenization of placentas with minimal settling, which aided in the uniformity of the placenta mixture and with the administration through the oral gavage apparatus. Additionally, we anticipated that the hormonally mediated effects of placentophagia could be related to steroid hormones, which are hydrophobic and therefore oil-soluble. California mice typically produce small litters of 1–4 pups [10,11], suggesting that treatment with only a single placenta is likely to be biologically relevant. One hour after treatment, we gave each mouse a pain-sensitivity test, followed by exploratory-behavior and paternal-responsiveness tests

beginning 4 h post-treatment (see below). Finally, we collected trunk blood immediately after subjects underwent the behavioral tests for analysis of circulating corticosterone concentrations in plasma as a measure of HPA activity.

2.3. Ethical note

All procedures used were in accordance with the *Guide for the Care and Use of Laboratory Animals* and were reviewed and approved by the University of California Riverside IACUC (protocol 20120033). The University of California Riverside is fully accredited by AAALAC.

2.4. Placenta procurement and administration

We collected near-term placentas as previously described [27] from the first-time gestating females cohabitating with males from the expectant condition. We determined approximate parturition dates by the presence of a sharp weight increase in females, which typically occurs 2–4 days prior to parturition (unpub. data). Near-term fetuses are also noticeable during this period on the ventrolateral abdominal area of pregnant mothers, and mothers' nipples increase in volume (unpub. obs). Near-term gestating females were euthanized by CO_2 inhalation, and the uterus was immediately dissected out and placed in a clean petri dish. Each fetus and its placental membranes were then freed from the uterine tissue using microscissors and forceps. Placental membranes were quickly detached from the fetus, and fetuses were immediately euthanized by an intra-peritoneal injection of 0.1 mL of pentobarbital (Fatal-Plus: Vortech Pharmaceuticals, Dearborn, Michigan, USA). Each individual placenta was weighed, placed into a 1 mL microcentrifuge tube, homogenized with a glass pestle in 0.1–0.2 mL of sesame oil, extracted with a sterile 1 mL syringe, and placed on ice.

We performed oral gavage using a 5 cm section of Silastic® laboratory tubing (1.57 mm inside diameter × 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 mice. The needle with tubing was then attached to either a syringe containing a single placenta (~0.4 g, 0.2–0.3 mL in volume) homogenized in sesame oil (total volume: 0.5 mL) or to a sterile 1 mL syringe containing 0.5 mL sesame oil alone. The average weight of near-term *P. californicus* placentas is ~0.40 g (unpub. data).

Between 08:30 and 10:00 h on the morning of testing, we isolated each mouse in a clean cage containing bedding, food, and water. Placentas were harvested between 08:00 and 09:30 h, and mice underwent oral gavage 30–180 min afterwards. We treated mice 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; [22]) 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 then returned to their isolation cages for recovery. The recovery time (i.e., time until mice were locomoting) from anesthesia was 1–3 min, at which point we observed animals in their isolation cages for 10 min before being returned to the colony room in the isolation cages. Placenta-treated males and their placenta donors were not pair mates and were no more closely related than second cousins.

2.5. Pain sensitivity

We measured pain sensitivity using a protocol developed for California mice by Zhao et al. [34], modified from one employed by others in lab mice and rats (e.g., [30,32]). Tests were performed between 10:00 and 13:00 h, 1 h after placenta or oil treatment. We chose 1 h because previous data on rats suggest that the hypoalgesic effect of placentophagia is detectable for up to 60 min (Abbott et al. [1]). A single male mouse was placed on a hot plate set at 44.0 (± 1.0) °C, and the latency for the mouse to show nociceptive behaviors (see below) was measured. A previous study indicated that this temperature was high enough to stimulate nociceptive behaviors in California mice without causing tissue damage, and was low enough to permit detection of inter-animal differences [34]. We performed tests in an environmental chamber with temperature and humidity maintained at 23 °C and 70%, respectively. Illumination was set to 1400 lx.

Ten to 20 min before each test, we moved individual mice in their isolation cages from the colony room to the environmental chamber. They were then placed on the hot plate, which was covered by a plexiglass cylinder (6 cm height × 20 cm diameter), and 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 (unpub.) revealed that California mice frequently lick their front paws, so only hind-paw behaviors were used as measures of nociception. Immediately after showing any of the above behaviors, we placed the mice back in their isolation cages and returned to the colony room. Mice that did not show any of these behaviors were removed from the hot plate after 120 s to prevent tissue injury. The hot plate was disinfected after each test. We considered mice to have lower pain sensitivity when they had longer latencies to show nociceptive behaviors.

2.6. Exploratory behaviors and paternal responsiveness

We characterized exploratory behaviors and paternal responsiveness using a modified open-field test (see below), beginning 4 h after placenta or oil administration (i.e., 3 h after pain-sensitivity tests). In a previous study we found that placenta-treated virgin males showed shorter latencies to approach a novel stimulus 1 h post-treatment, compared to oil-treated controls, and that placenta treated virgin-male mice show reduced activity in brain nuclei regulating neophobic responses for up to 4 h after treatment (Perea-Rodriguez, unpub. Ph.D. dissertation).

The open-field arena was a 1.0 m × 1.0 m square with a height of 0.48 m, constructed of opaque black plastic, placed on top of a clean sheet of white butcher paper to enhance contrast between the arena floor and the darkly colored mice. The inner sides of the arena walls were sanded down to prevent glare or reflection that might distract the mice. Tests were recorded by a digital camera above the arena. After each test, the arena was disinfected and the butcher paper replaced. The arena was located in an environmental chamber maintained at 1400 lx with two overhead white lights; temperature and humidity were maintained at 23 °C and 70%, respectively. For each test, we initially placed the male subject in the center of the arena and video-recorded it for 10 min (open-field test), at which point we placed a 1- to 4-day-old, unrelated pup in the center of the arena for an additional 10 min (paternal-responsiveness test). Males were decapitated within 1 min after the paternal-responsiveness test, and plasma was collected and assayed for corticosterone (see below).

We quantified exploratory behaviors 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. Using the software, the arena was divided into two concentric regions: an inner square, measuring 0.5 × 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. Mice proceeded to move around and explore the open field immediately after being placed in it. The latency to cross the center of the arena while exploring the arena, the total distance moved, the number of times a mouse crossed between the inner and outer regions (i.e., crossing bouts), and the duration of time spent in the inner square were determined for each mouse for the initial 10 min of testing, prior to introduction of the pup into the arena. We considered mice with longer distances traveled, greater total durations in the inner region, shorter latencies to cross the center of the open field while exploring the open-field, or higher numbers of crossing bouts to have lower anxiety [7].

The behavioral response of adult male mice to pups within the open-field arena was quantified using JWatcher software [3]. For the 10-min paternal-responsiveness test we measured latencies to approach pups, latencies to care for pups, and duration of caretaking behaviors (i.e., huddling pup, licking pup) [6].

2.7. Plasma corticosterone concentrations

We determined plasma corticosterone levels in blood samples collected from each mouse immediately after the open-field and paternal-responsiveness test. We assayed corticosterone using a commercially available double-antibody radioimmunoassay kit (07120103; MP Biomedicals, Solon, OH, USA) previously validated for use in California mice [5]. The assay standard curve was extended down from 25 to 12.5 ng/mL (90–91% bound) and went to 1000 ng/mL (19% bound). Samples were initially diluted at 1:400. Several values fell outside the range of the standard curve and were re-run at dilutions up to 1:1600 to ensure that interpolated values were valid; these values although high, fall within the natural range for the species [12]. Initial dilutions were chosen due to expected baseline corticosterone levels around the time of day of testing [12]. All samples were run in duplicate, and obtained values were considered usable if the corticosterone concentration fell within the curve and CVs were < 10%. Intra- and inter-assay CVs, calculated using an in-house plasma pool, were 5.50% and 6.03%, respectively.

2.8. Statistical analyses

Analyses were performed using R statistical software (R [28]). We tested behavioral and endocrine data for normality using Shapiro-Wilk tests, and for homogeneity of variance using Bartlett's tests. Non-normal data were log transformed (i.e., behavioral responses to pups and plasma corticosterone concentrations) or square-root transformed (i.e., pain sensitivity, exploration of the open field) before being analyzed parametrically. Corticosterone concentrations were analyzed by analysis of covariance, with time of day as a covariate to control for circadian changes in corticosterone secretion [12]. All behavioral outcome variables were analyzed using 2-way analyses of variance with treatment (i.e., oil or placenta), reproductive condition (i.e., virgin, expectants, or fathers) and their interaction as predictor factors. The analyses and predictions mentioned above were selected a priori. The alpha value was set at 0.05.

To determine if the behavior of mice in the open field was linked to their parental responsiveness or to their peripheral corticosterone levels, we conducted Spearman's correlations using untransformed data between total distance traveled during the initial 10-min in the open field and paternal response (latency to approach pup, latency to care for pup, and time spent caring for pup), as well as separate correlations between total distance traveled and plasma corticosterone levels. We performed correlational analyses separately for virgins, expectants, and fathers to avoid any confounding effects of reproductive condition on our outcome variables. A total of 5 pain-sensitivity tests and 4 plasma samples were unusable due to an apparatus malfunction. The resulting sample sizes, as well as means, standard errors, and statistical results for each measure, are shown in Table 1. Non-transformed data are presented in figures and tables for ease of interpretation.

3. Results

3.1. Pain sensitivity

Latencies to show nociceptive behaviors on the hot plate did not differ between placenta- and oil-treated mice or between fathers, first-time expectant males, and virgin males, nor was it affected by a treatment x reproductive condition interaction (Table 1).

3.2. Exploratory behaviors

Placenta-treated males traveled longer distances during the open-field test than did oil-treated males (ANOVA: main effect of treatment - $F_{1,\ 42} = 7.9$, $P = .01$), independent of reproductive condition or a treatment × reproductive condition interaction (Table 1, Fig. 1). Neither placenta treatment, reproductive condition, nor their interaction affected males' latencies to cross the center of the open-field arena, duration of time spent in the inner region of the arena, or number of crossing bouts (Table 1).

3.3. Parental responsiveness

Oil- and placenta-treated mice did not differ in their latencies to approach pups, latencies to care for pups, or in their overall duration of caretaking behaviors. On the other hand, we found a significant effect of reproductive condition on latencies to approach pups (ANOVA: main effect of reproductive condition; $F_{2,\ 41} = 3.75$, $P = 0.03$;), as well as latencies to care for pups (ANOVA: main effect of reproductive condition; $F_{2,\ 41} = 17.74$, $P < 0.001$;): fathers had shorter latencies to care for pups, compared to both expectants (Tukey's HSD test: $P < 0.001$) and virgins (Tukey's HSD test: $P < 0.001$; Fig. 2). Additionally, fathers spent more time performing caretaking behaviors toward pups (main effect of reproductive condition: $F_{2,\ 41} = 9.41$, $P < 0.001$; ANOVA), when compared to both expectants (Tukey's HSD test: $P < 0.001$) and virgins (Tukey's HSD test: $P < .001$; Fig. 2). Of the 15 virgin males tested, only one placenta-treated male and no oil-treated males displayed caretaking behavior toward pups. In comparison, seven expectant males (3/8 placenta-treated, 4/8 oil-treated), and 12 first-time fathers (7/8 placenta-treated, 5/8 oil-treated) performed caretaking behavior. The interactions between placenta or oil treatment and reproductive condition had no effect on latency to approach pups, latency to care for pups, or overall duration of caretaking behaviors.

3.4. Plasma corticosterone concentrations

Peripheral corticosterone levels immediately after the exploratory and parental-responsiveness tests were not affected by placenta treatment and did not differ between virgins, first-time expectant males, and fathers. Moreover, corticosterone concentrations were not influenced by an interaction between treatment and reproductive condition.

3.5. Correlations between exploratory behaviors and parental behaviors

To avoid any effects of the individual's reproductive condition to

Table 1
Latencies to show nociceptive behaviors, exploratory behavior during a 10-min open-field test, pup-directed behavior during a 10-min paternal-responsiveness test in an open field, and post-test plasma corticosterone levels of virgin males, expectant first-time fathers, and first-time fathers treated orally with either conspecific placenta in oil or oil alone (n = 6–8 per treatment per reproductive condition). Data are presented as non-transformed means and standard errors, and statistical results are from treatment × reproductive condition ANOVAs, or ANCOVA with time of day as a covariate for corticosterone data.

Measure	Virgins	Expectants	Fathers	Main effect of treatment	Main effect of repro. condition	Treatment × repro. condition interaction
Latency to nociception (s)	Oil: 29.47 ± 6.79 n = 8	Oil: 15.21 ± 2.80 n = 5	Oil: 40.41 ± 17.55 n = 8	$F_{1, 37} < 1.00$	$F_{2, 37} < 1.00$	$F_{2, 37} < 1.00$
	Placenta: 17.92 ± 5.28 n = 7	Placenta: 26.58 ± 8.24 n = 7	Placenta: 42.77 ± 14.36 n = 8	P = .90	P = .54	P = .37
Latency to cross centre of open field (s)	Oil: 242.2 ± 64.6 n = 8	Oil: 341.60 ± 92.93 n = 8	Oil: 340.80 ± 90.22 n = 8	$F_{1, 42} < 1.00$	$F_{2, 42} < 1.00$	$F_{2, 42} < 1.00$
	Placenta: 411.62 ± 70.35 n = 8	Placenta: 307.71 ± 74.21 n = 8	Placenta: 262.56 ± 86.94 n = 8	P = .87	P = .98	P = .23
Duration Inside Inner 50% of open field (s)	Oil: 575.6 ± 7.09 n = 8	Oil: 575.20 ± 11.08 n = 8	Oil: 576.1 ± 5.44 n = 8	$F_{1, 42} < 1.00$	$F_{2, 42} < 100$	$F_{2, 42} < 1.00$
	Placenta: 583.90 ± 2.82 n = 8	Placenta: 574.90 ± 8.77 n = 8	Placenta: 563.7 ± 17.44 n = 8	P = .66	P = .88	P = .22
Number of crossing bouts in open field (s)	Oil: 44.62 ± 13.87 n = 8	Oil: 27.88 ± 9.96 n = 8	Oil: 23.75 ± 6.98 n = 8	$F_{1, 42} < 1.00$	$F_{2, 42} < 1.00$	$F_{2, 42} < 1.00$
	Placenta: 27.00 ± 5.13 n = 8	Placenta: 43.25 ± 16.03 n = 8	Placenta: 36.88 ± 6.77 n = 8	P = .47	P = .89	P = .26
Total distance Traveled in open field (m)	Oil: 43.67 ± 9.49 n = 8	Oil: 50.03 ± 10.26 n = 8	Oil: 39.66 ± 8.58 n = 8	$F_{1, 42} = 7.90$	$F_{2, 42} < 1.00$	$F_{2, 42} < 1.00$
	Placenta: 49.37 ± 5.85 n = 8	Placenta: 84.01 ± 15.39 n = 8	Placenta: 76.34 ± 14.71 n = 8	**P = .01**	P = .28	P = .52
Latency to approach pup (s)	Oil: 251.00 ± 102.51	Oil: 294.62 ± 93.05	Oil: 161.41 ± 95.73	$F_{1, 41} < 1.00$	$F_{2, 41} = 3.75$	$F_{2, 41} < 1.00$
	Placenta: 373.79 ± 109.62	Placenta: 145.51 ± 69.77	Placenta: 24.55 ± 12.91	P = .35	**P = .03**	P = .27
Latency to caretaking behavior (s)	Oil: 600 ± 0.00 n = 8	Oil: 387.24 ± 85.72 n = 8	Oil: 254.37 ± 101.64 n = 8	$F_{1, 41} < 1.00$	$F_{2, 41} = 17.74$	$F_{2, 41} < 1.00$
	Placenta: 527.33 ± 72.66 n = 8	Placenta: 426.06 ± 85.15 n = 8	Placenta: 66.20 ± 19.53 n = 8	P = .18	**P < .001**	P = .30
Duration of caretaking behavior (s)	Oil: 0.00 ± 0.00 n = 8	Oil: 76.96 ± 42.34 n = 8	Oil: 245.93 ± 75.26 n = 8	$F_{1, 41} < 1.00$	$F_{2, 41} = 9.41$	$F_{2, 41} < 1.00$
	Placenta: 54.33 ± 54.33 n = 8	Placenta: 90.82 ± 53.87 n = 8	Placenta: 209.17 ± 52.09 n = 8	P = .53	**P < .001**	P = .85
Plasma corticosterone (ng/ml)	Oil: 2210.00 ± 299.90 n = 8	Oil: 2183.90 ± 317.30 n = 7	Oil: 2197.00 ± 161.50 n = 8	$F_{1, 37} < 1.00$	$F_{2, 37} < 1.00$	$F_{2,37} < 1.00$
	Placenta: 2049.00 ± 131.10 n = 8	Placenta: 2280.00 ± 189.00 n = 6	Placenta: 2028.00 ± 188.50 n = 7	P = .72	P = .66	P = .72

Significant p-values are presented in bold.

our experimental paradigm we performed Spearman's correlations separately for each reproductive condition, using pooled data from placenta- and oil-treated mice. We found no significant associations between total distance traveled during the first 10 min in the open-field arena and latencies to care for pups by virgins ($\rho = -0.30$, P = .26, n = 16), expectants ($\rho = -0.14$ P = .58, n = 16), or fathers ($\rho = -0.31$ P = .22, n = 16). Similarly, we found no relationship between total distances traveled during the initial 10-min open-field test and total durations of caretaking behaviors (virgins: $\rho = 0.30$, P = .26, n = 16; expectants: $\rho = 0.19$, P = .47, n = 16; fathers: $\rho = -0.09$, P = .72, n = 16). We did find a marginally significant negative correlation between total distance traveled and latency to approach a pup for oil-treated virgin males ($\rho = -0.49$, P = .05, n = 16), but this relationship was not found in expectants ($\rho = -0.45$, P = .08, n = 16) or fathers ($\rho = -0.30$, P = .25, n = 16).

3.6. Correlations between behaviors and corticosterone concentrations

Correlations of data pooled across all three reproductive conditions showed no relationship between total distance traveled and post-test plasma corticosterone levels for any of the reproductive conditions studied (virgins: $\rho = -0.32$, P = .22, n = 16; expectants: $\rho = -0.26$, P = .38, n = 12; fathers: $\rho = -0.09$, P = .72, n = 14), between latency to care for pups and post-test plasma corticosterone level (virgins: $\rho = 0.37$, P = .17, n = 16; expectants: $\rho = 0.47$, P = .10, n = 12; fathers: $\rho = 0.07$, P = .78, n = 14), or between the total duration of

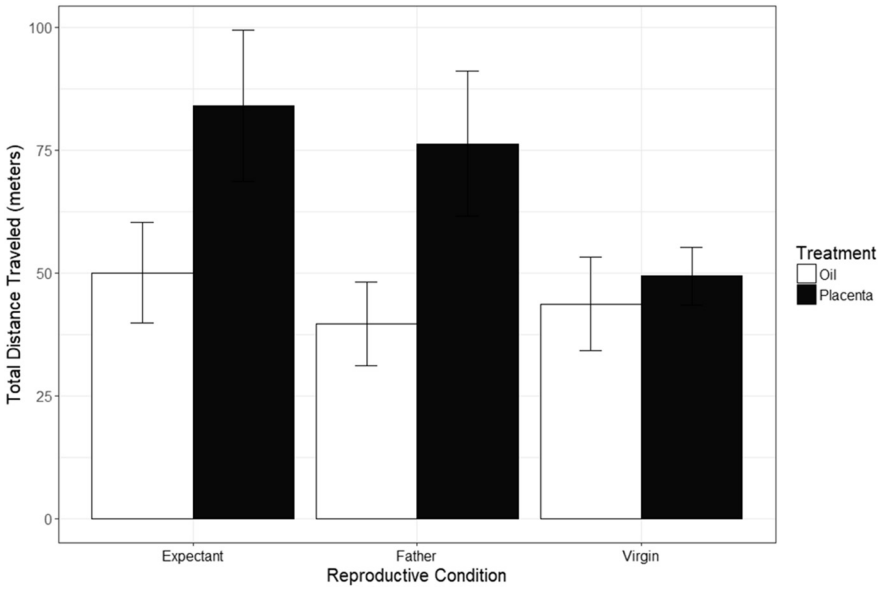

Fig. 1. Total distance traveled by virgin males, expectant first-time fathers, and first-time fathers, treated orally with either conspecific placenta in oil (white circles and bars) or oil alone (black circles and bars; n = 8 per treatment per reproductive condition) during a 10-min open-field test. Total distance traveled was significantly higher in placenta-treated males than in oil-treated males, independent of reproductive condition.

caretaking behaviors and post-test plasma corticosterone level (virgins: $\rho = -0.37$, P = .17, n = 16; expectants: $\rho = -0.50$, P = .07, n = 12; fathers: $\rho = 0.15$, P = .58, n = 14).

4. Discussion

In this study, we investigated the possible effects of placentophagia on pain sensitivity, exploratory behaviors, paternal responsiveness, and plasma corticosterone concentrations in male California mice. Additionally, we aimed to identify the possible influences of reproductive condition on effects of placentophagia. We found that oral treatment with placenta increased the exploratory behavior of male mice (i.e., total distance traveled during a 10-min open-field test), independent of their reproductive condition. Furthermore, we found that our modified open-field paradigm, which we used to measure paternal motivation under presumably anxiogenic conditions, yielded differences in latencies to approach and care for pups, as well as differences in durations of caretaking behavior, across reproductive conditions, but not between placenta and oil treatments. Specifically, we found that first-time fathers approached and cared for pups more quickly and spent more time caring for pups, when compared to both first-time expectant fathers and virgin males. Finally, neither placenta treatment nor reproductive condition affected pain sensitivity or plasma corticosterone concentrations following the open-field test and paternal responsiveness test.

Studies on the causes and consequences of maternal placentophagia in mammals suggest that a major benefit of ingesting the afterbirth is to increase mothers' pain threshold via changes in opioid-mediated analgesia, which may benefit both mothers and neonates [19]. This hypoalgesic effect is also found in adult male rats after placenta ingestion, and has a rapid onset (~1 min) that can last for up to 60 min (Abbott et al. [1]). 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 1 hour after treatment with placenta. We found no effect of reproductive condition on latencies for mice to show nociceptive behaviors. It is possible, however, that placenta ingestion did modulate pain sensitivity in our study, but that this effect had dissipated by the time we performed the hot-plate test. Alternatively, it is possible that the changes in pain sensitivity previously reported in male California mice due to the pair-bonding process may be mediated by non-opioid mechanisms [4,24], and thus explain why placentophagia did not result in changes in pain sensitivity in our current study.

In a separate study, we found that adult virgin male California mice that were administered a near-term placenta via oral gavage showed reduced latencies to approach a novel stimulus (i.e., decreased neophobia), as well as reduced neural activity (Fos-immunoreactivity) in the dorsal area of the bed nucleus of the stria terminalis (BST), when compared to adult virgin males administered oil vehicle (Perea-Rodriguez, unpub. Ph.D. dissertation). These findings suggest that virgin males undergo behavioral and neural 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-like responses [31]. Results of the present study are consistent with this possibility: increased exploratory behavior in an open field, as we observed in placenta-treated males, regardless of reproductive condition, is typically interpreted as indicative of low anxiety [7]. 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 male caretaking behaviors toward experimentally presented pups.

We did not find any differences in exploratory behaviors in the open field among fathers, expectant fathers, and virgin males, and our analyses did not reveal any significant correlations between exploratory behaviors and parental responsiveness. In other studies, we have found negative correlations between anxiety-related behavioral or neural measures and indices of paternal responsiveness in male California mice [5,6]. Several studies comparing behavioral and neural markers of anxiety between California mouse fathers and non-fathers have yielded mixed results [5,13,16]. Thus, to date, neither the effects of fatherhood on anxiety nor the relationship between anxiety and paternal responsiveness in individual males are well understood.

Some important caveats should be kept in mind when interpreting the results of this study. First, the oral gavage procedure by which we administered placenta eliminated any possible effects that placenta and amniotic fluid might have had via olfactory or accessory olfactory pathways, and the oil preparation used may have limited absorption of some of the biologically active substances found in placenta and

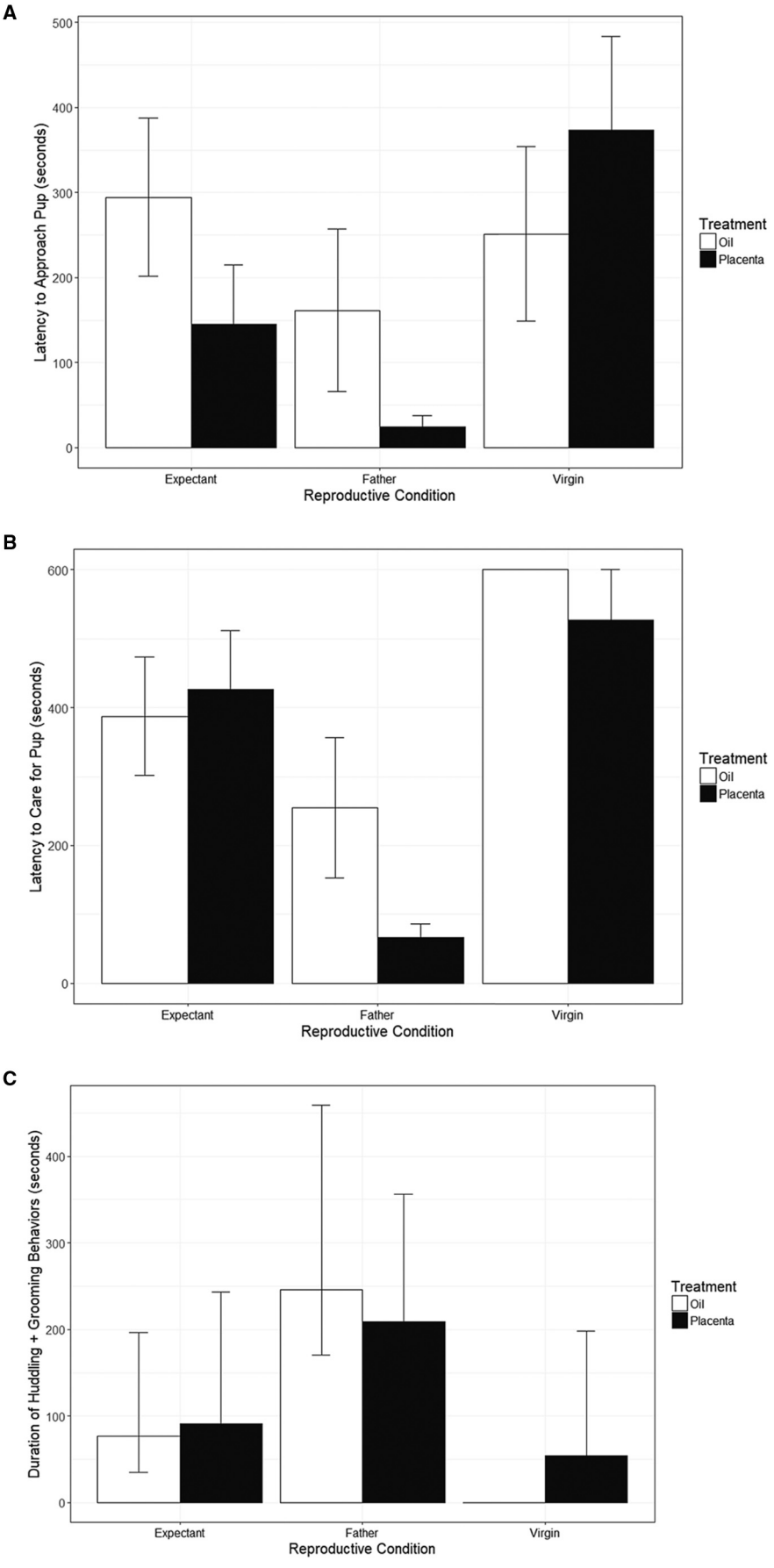

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Fig. 2. Behavioral responses to an unfamiliar pup, presented in an open field for 10 min of virgin males, expectant first-time fathers, and first-time fathers, treated orally with either conspecific placenta in oil (white bars) or oil alone (black bars; n = 8 per treatment per reproductive condition). Latency to approach pups (A), latency to care for pups (B), and total duration of time spent caring for the pup (C) were not influenced by treatment but differed among reproductive condition. First-time fathers showed significantly shorter latencies to approach pups, as well as shorter latencies to care for pups and more time spent caring for pups, compared to expectants and virgins. See text for details.

amniotic fluid. Second, the near-term placentas that we used might not have contained all of the hormones or other active components, or different amounts of these, compared to full-term placentas. Finally, our samples sizes are somewhat small for a behavioral study.

5. Conclusions

In conclusion, this study sought to characterize the consequences of placentophagia in males of a monogamous and biparental mammal. Our results are consistent with our previous findings that ingestion of placenta may have anxiolytic effects in males but may not directly influence their motivation to engage in caretaking behavior toward pups. Further studies should investigate the hormonal and neural mechanisms underlying the anxiolytic effect of placentophagia, the specific components of placenta that trigger this effect, and its potential functional significance.

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