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FULL PAPER

Toxicology

Effect of the neonicotinoid pesticide clothianidin at a no-observed-adverse-effect-level (NOAEL) dose on maternal behavior in pregnant mice and their female offspring

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ABSTRACT. Our previous reports showed that exposure to the neonicotinoid pesticide clothianidin (CLO) at a no-observed-adverse-effect-level (NOAEL) dose during fetal development and lactation in mice led to higher rates of maternal neglect and infanticide. Although the demonstrated association between decreased oxytocin secretion and decreased maternal parenting behavior implies a link to declining oxytocin levels, no evidence has yet emerged in CLO to clearly establish such an association. This study investigated the effects of CLO on maternal behavior and oxytocin in C57BL/6N mice exposed during pregnancy and lactation (F0 mothers) as well as in their adult female offspring (F1 mothers). The effects were assessed using nest building assays during pregnancy and pup retrieval assessment after delivery. The results showed a decrease in oxytocin secretion and a marked decrease in pup retrieval behavior among the F0 mothers in the CLO exposure group compared to those in the control group. Their offspring, the F1 mothers, showed significantly lower nest-building scores during pregnancy. In conclusion, this study is the first to examine the potential mechanisms by which CLO exposure in mothers at the NOAEL dose during pregnancy and lactation results in reduced plasma oxytocin levels, subsequently leading to a decline in maternal behaviors such as pup retrieval. Furthermore, these effects may impair maternal behaviors in the next generation, when the offspring mice become mothers.

KEYWORDS: clothianidin, maternal behavior, nest building, oxytocin, pup retrieval

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INTRODUCTION

Neonicotinoid pesticides (NNs), structurally similar to nicotine, have a strong affinity for nicotinic acetylcholine receptors (nAChRs) in insects and have been considered less toxic to mammals. However, it is now clear that NNs, even at or below the no-observed-adverse-effect levels (NOAELs) have adverse effects on the reproductive systems [13, 16, 22, 39, 46] and nervous systems [9–11, 13–15, 23, 25, 29, 37, 38, 47] of birds and mammals. In addition, it was reported that clothianidin (CLO), a type of NN, and its metabolites transfer rapidly from the mother to the fetus through the placenta [12, 30] and that CLO is metabolized and concentrated

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in the mother and also transfers rapidly into breast milk [36]. Furthermore, NNs were detected in Japanese adults, children, and newborns, in addition to organophosphorus and pyrethroid [17, 18, 32, 42]. CLO ingestion in pregnant and lactating mother mice can alter the behaviors [25, 34, 35] as well as the reproductive systems [22, 46] and immune systems [27] of their offspring over generations. Therefore, a risk assessment of NN toxicity that takes into account multigenerational and transgenerational effects is urgently needed. More recently, environmental chemicals have been shown to exert transgenerational effects by promoting epigenetic mutations through germ cells. For example, fetal rats exposed to the herbicide glyphosate exhibited an increased health risk in the F2 and F3 generations and differentially methylated regions of sperm DNA [24]. Moreover, exposure to nicotine during pregnancy induced behavioral abnormalities in the F1 and F2 generations, with DNA hypomethylation assumed to be the contributing factor [3].

Our previous studies showed that exposure of mice to NNs during the fetal and lactation periods increased neglect and infanticide across generations and altered maternal behaviors [22, 35]. It has been shown that nurturing behavior is diminished in oxytocin receptor (RAGE) KO mice [45]. Optogenetic activation in the paraventricular hypothalamic area (PVN) prompted maternal behaviors and increased plasma oxytocin levels [19]. Given that oxytocin plays crucial roles in maternal behaviors, the reduction of plasma oxytocin levels in rats exposed to cigarette smoke during lactation suggests that women who smoke during pregnancy may subsequently breastfeed their children less frequently and for a shorter period [8, 28]. Furthermore, it is known that offspring raised without sufficient nurturing by their mothers will similarly neglect their own offspring [4]. However, no reports clearly show that exposure to NNs leads to reduced oxytocin secretion.

The hypothalamus–pituitary–adrenal (HPA) axis has also been associated with maternal behaviors. In a previous study, when pregnant mice were given CLO for consecutive days, CLO accumulated only in the adrenal gland [12]; thus, it is important to assess adrenal toxicity. It was reported that exposure to imidacloprid, a type of NN, disrupts the regulatory mechanism of the HPA axis in rats [1], revealing the toxicity of NNs to the HPA axis. Chronic psychosocial stress during pregnancy caused dysregulation in HPA axis function, increased adrenal weight, and abnormalities in maternal behaviors in postpartum female mice [48]. Adrenalectomy in rats during late pregnancy reduced maternal behaviors including licking the pups, crouching over the pups, and spending time in the nest which were restored by postpartum subcutaneous implantation of corticosterone pellets [40]. Furthermore, the frequency of maternal licking and grooming may alter gene expression and the stress responsiveness of the HPA axis in offspring [6], whose HPA axis-regulating function is affected by the quality of parental nurturing behaviors.

Therefore, to clarify how NNs affect oxytocin and maternal behaviors (specifically, nurturing behaviors), we evaluated maternal behaviors through nest building assays during pregnancy and pup retrieval assessments after delivery, while measuring plasma oxytocin levels using liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). We also investigated whether the effects of NN-induced aberrations in maternal behaviors extend to the next generation.

MATERIALS AND METHODS

Experimental animals

Pregnant C57BL6/NCrSlc mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained in individual ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) measuring 40.5 × 20.5 × 18.5 cm and were kept under controlled conditions of temperature (23 ± 2°C), humidity (50 ± 10%), and ventilation (cage: 75 times/hr) on a 14-hr light/10-hr dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to a pellet diet (DC-8; Clea Japan, Tokyo) and water. This study was approved by the Institutional Animal Care and Use Committee (Permission #30-01-01, #2023-05-01) and carried out according to the Kobe University Animal Experimental Regulations.

The mothers were divided into two groups (F0 Control: 10 mothers, F0 CLO-NOAEL: 6 mothers, F1 Control: 6 mothers, F1 CLO-NOAEL: 8 mothers). CLO (95% purity [13]) was administered as described previously [25, 35]. Briefly, it was administered to the mothers at a volume of 0 (Control group) or 65 mg/kg body weight (CLO-NOAEL group) from gestational day (GD) 1.5 to postnatal day (PD) 28–30 with reference to the NOAEL (ICR female mice: 65.1 mg/kg/day) [7, 43]. Rehydration gel (MediGel® Sucralose; ClearH_2O, Portland, ME, USA) with vehicle (1% dimethyl sulfoxide: DMSO) or CLO was used for administration. To verify the amount of gel ingested, the remaining gel was weighed each time it was replaced. We also weighed the mothers and pups once a week. To standardize milk volume, a maximum of 6 pups per litter were randomly selected on PD3, and litters with fewer than 3 pups were excluded from the experiment. When the F1 offspring reached 12 weeks of age, males and females within the same group were arbitrarily paired with each other, and the pregnant female mice were used as F1 mothers.

When the offspring of mice mated on the same day reached PD28-30, the F0 and F1 mothers were deeply anesthetized with isoflurane using an anesthesia apparatus (BS-400T; Brain Science Idea, Osaka, Japan) the moment they left their cages, and blood was collected from the heart. Mothers and pups lived together until we weaned the pups. The mothers were then euthanized, and the adrenal glands and brain (including the pituitary gland) were extracted (Fig. 1).

Nest building assay

Nest building assays were conducted on GD10.5 and GD17.5 (Fig. 1). On the day before the experiment, 3.0 g of cotton was placed in a fixed position in the cage (specifically, the left corner of the front side of the cage) at 7:00 p.m. Nest-building behavior was then assessed at 9:00 a.m. the following day. Three criteria, adapted from previous reports [5, 26], were employed: the amount of shredding nestlet; the quality of the nest (height and shape); and the usage of the nestlet material, each scored out of 2, 5, and 2, respectively. The sum of these three criteria (resulting in a maximum score of 9) was used to determine the nest-building scores.

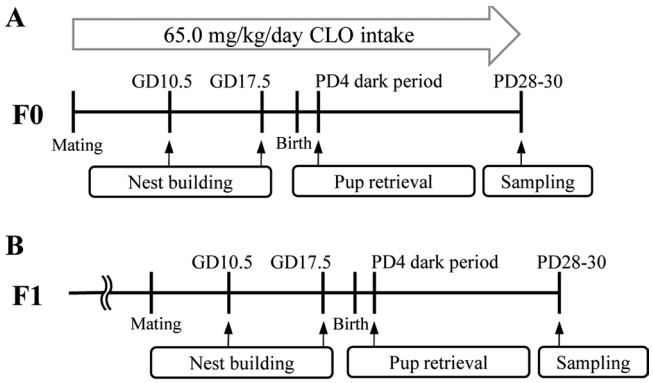

Fig. 1. Schematic diagram of the experimental design of the present study. (**A**) F0 mothers. Clothianidin (CLO), a type of neonicotinoid pesticide (NN), was fed *ad libitum* to pregnant mice (C57BL/6N) from gestational day (GD) 1.5 to postnatal day (PD) 28-30 [65 mg/kg/day: no-observed-adverse-effect level (NOAEL) in the pesticide evaluation report]. Their male and female offspring within the same group were arbitrarily mated with each other, and the pregnant female mice were used as (**B**) F1 mothers. Maternal behaviors were evaluated by conducting nest building assays at GD10.5 and GD17.5 and a pup retrieval assessment in the dark period of PD 4. In PD28-30, blood, adrenal gland, and brain (including pituitary gland) were collected from the mothers. The number of mothers in each group was as follows: 10 F0 Control, 6 F0 CLO-NOAEL, 6 F1 Control, and 8 F1 CLO-NOAEL mothers.

Pup retrieval assessment

Pup retrieval was assessed during the dark period of PD4 (Fig. 1). The experimental procedure followed previous reports [19, 26]. Prior to the start of the test, the mother was removed from the home cage and placed in a waiting cage. Of her 6 pups, 4 were randomly placed outside the nest equidistant apart, facing away from the nest, while the remaining 2 stayed in the nest. After 5 min of separation, the mother was reintroduced into the home cage and allowed to retrieve the pups. Retrieval was defined as "a mother grasping her pup by the dorsal neck with her mouth and returning it to the nest". If a mother did not return the pup to the nest, retrieval was considered incomplete. Retrieval behavior was video recorded for 6 min, and the latency to retrieve (the number of seconds it takes a mother to retrieve the first pup) was measured. Completion rate was calculated by dividing the number of the mothers who retrieved all pups (4 pups) by the total number of the mothers.

Measurement of plasma oxytocin levels

Blood samples were centrifuged (3,000 × *g*, 10 min, 4°C) to separate the plasma, and plasma oxytocin was measured by LC-ESI/MS/MS. To a 1.5 mL tube were added and vortexed 100 µL of serum, 900 µL of acetonitrile, and 100 µL of 100 ppb Oxytocin-D5 as an internal standard. The tubes were then centrifuged at 10,000 × *g* for 10 min, and 900 µL of the supernatant was transferred to a new 1.5 mL tube. After concentration and drying, the solution was then re-dissolved in 100 µL of 30% methanol solution, and the target substances were measured by LC-ESI/MS/MS. The analytes were detected by electrospray ionization (ESI) in positive ion mode. An Agilent 1290 Infinity II HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used for LC, and an Agilent 6495B triple quadrupole mass spectrometer (Agilent Technologies) was used for MS as described elsewhere [10].

Statistical analyses

Statistical analyses were performed using BellCurve for Excel (Version 4.05; SSRI, Tokyo, Japan). Both the behavioral data and plasma oxytocin levels were analyzed using Welch's *t*-test and the Mann-Whitney *U*-test. The Smirnov-Grubbs two-tailed test was used to identify and exclude outliers, while the Kolmogorov-Smirnov test was used to assess normality. The results were considered significant when the *P*-value was less than 0.05. Since we observed no statistical difference in the results on any day within PD28-30, we analyzed the data as one group.

RESULTS

CLO did not affect general health status

There was little individual variation in the daily gel intake of F0 mothers, with no significant differences between the Control and CLO-NOAEL groups. We confirmed that the presence or absence of CLO was not associated with gel preference or gel intake (Supplementary Fig. 1).

For both F1 and F2 pups, no significant effects of CLO were observed regarding litter size, pup sex distribution, and pup birth weight (Supplementary Fig. 2). In F0 mothers, one case of infanticide was observed in each group (Supplementary Fig. 3). In F1 mothers, there was one case of infanticide only in the Control group (Supplementary Fig. 3).

No significant weight change effects due to CLO were observed in F0 mothers and F1 pups (Supplementary Fig. 4A). There were no significant differences in body weight for either F1 mothers or F2 pups (Supplementary Fig. 4B).

CLO exposure significantly inhibited pup retrieval and decreased oxytocin levels in F0 mothers

Nest-building scores were significantly higher in the CLO-NOAEL group than in the Control group at GD10.5 (P=0.0262) (Fig. 2A, Supplementary Fig. 5). Although there was no significant difference in scores at GD17.5, the CLO-NOAEL group built their nests more thoroughly (Fig. 2A, Supplementary Fig. 6). In the pup retrieval assessment, latency to retrieve was significantly longer in the CLO-NOAEL group (P=0.0333) (Fig. 2B). Only one mother (17%) in the CLO-NOAEL group retrieved all 4 pups within 6 min, compared to 5 mothers (50%) in the Control group (Fig. 2C). Adrenal weight was significantly lighter (P=0.0466) (Fig. 2D), and the plasma oxytocin level tended to be lower in the CLO-NOAEL group (P=0.0526) (Fig. 2E).

CLO exposure significantly inhibited nesting, but had little effect on pup retrieval or oxytocin in F1 mothers

Nest-building scores were significantly lower in the CLO-NOAEL group for both GD10.5 and GD17.5 (GD10.5: P=0.0087, GD17.5: P=0.0236) (Fig. 3A), with the CLO-NOAEL group rarely constructing nests (Supplementary Figs. 7 and 8). In the pup retrieval assessment, there were no significant differences in either the latency to retrieve or the completion rate of pup retrieval (Fig. 3B and 3C). Furthermore, no significant differences were observed in adrenal weight and plasma oxytocin level (Fig. 3D and 3E).

DISCUSSION

Results from the nest-building assay and pup retrieval assessment differed considerably between F0 and F1 mothers. In particular, F0 mothers showed more active nest-building behavior than F1 mothers, a surprising divergence from our initial expectations. Our previous reports showed that subchronic exposure to CLO in mice resulted in anxiety-like behaviors [14, 15, 29]. Therefore, the heightened anxiety levels might have influenced the meticulous nest-building behavior observed in F0 mothers. In contrast, F1 mothers showed a marked decline in nest-building behavior. A previous study found that exposure to chlorpyrifos, a type of organophosphorus pesticide, during lactation reduces the motivation for nest-building behavior among offspring when they become mothers [44]. Organophosphorus pesticides inhibit acetylcholine-degrading enzyme, leading to the accumulation of excessive acetylcholine. As a result, the surplus acetylcholine binds to the nAChR receptors, leading to organophosphorus compounds that, like CLO, exhibit neurological effects. F1 mothers ingested CLO not only during lactation but also in the fetal period. It is assumed that the more severely damaged nest-building behavior of F1 mothers is attributable to this greater exposure during their development. In addition, as indicated by the pup retrieval behavior of F0 mothers, the CLO-NOAEL group of F0 mothers showed significantly impaired nurturing behaviors. It is possible that this poor nurturing behavior of the F0 parents was passed on to the F1 mothers, thereby exacerbating the damage to the nest-building behavior of the latter.

Then, the CLO-NOAEL group of F0 mothers exhibited significantly longer retrieval latency, and the blood oxytocin level measured at weaning tended to be lower, aligning with our expectations. C57BL/6N mice, the experimental animals in this study, grow more slowly than other strains and reach sexual maturity at around 12 weeks of age. Therefore, the pups were weaned at 4 weeks after birth, and the plasma oxytocin level in mothers was measured at that time. Given that pup retrieval was assessed at PD4, during the peak of lactation, it is possible that the blood oxytocin level in F0 mothers at this juncture was also low due to the impact of CLO. According to previous reports, oxytocin secretion is necessary for the induction of nurturing behaviors, since the destruction of PVN, where many oxytocin neurons reside, and infusing an oxytocin antagonist into the preoptic area (POA) to female rats after parturition inhibited the development of nurturing behaviors [31]. The observed low plasma oxytocin levels in F0 mothers can be considered to suggest that oxytocin is important for the development and maintenance of nurturing behaviors [20, 33]. These findings imply that exposure to CLO led to a reduction in oxytocin levels, leading to a notable increase in the latency to initiate retrieval of the first pup. In addition, previous reports have shown that mothers retrieve in response to ultrasonic vocalizations (USV) emitted by their pups [41] and that exposure of 1- to 4-day-old rat pups to chlorpyrifos inhibits USV [2]. In other words, the significant delay in F0 mothers' pup retrieval may have resulted from CLO exposure inhibiting the USV emitted by the pups. It is also presumed that factors related to the pups themselves played a crucial role in this behavior. These findings suggest that CLO exposure affects both mothers and pups, leading to the inhibition of retrieval. On the other hand, F1 mothers did not ingest CLO when they themselves were pregnant or lactating, and thus their plasma oxytocin levels were not reduced. In the same way as F1 mothers, the F2 pups did not exhibit inhibited USV. Therefore, it was assumed that F1 mothers were able to initiate pup retrieval more easily.

The reduced weight of adrenal glands in F0 mothers suggested adrenal toxicity caused by CLO. This may have been accompanied by an abnormality in the regulatory system of the HPA axis, leading to changes in maternal behavior. According to previous reports, adrenalectomy reduced maternal behaviors in pregnant rats [40]. Given this, the decrease in maternal behaviors associated with the decrease in adrenal weight of F0 mothers observed in this study may be due to changes in plasma corticosterone levels. However, these effects were not elucidated. Further research is needed.

Our previous studies observed maternal neglect and infanticide by CLO-exposed mothers in the F1 generation [22, 35], but the present study did not. The reason for this difference is not clear, but it may be due to differences in experimental conditions between the studies. For example, weaning the F1 pups at 4 weeks avoided the stress of early separation and ensured adequate nurturing by the F0 mothers. It is known that early-weaning manipulation deprives offspring of a certain level of maternal care, and as a consequence, the offspring show higher anxiety levels and lower maternal behaviors in their own adulthood [21]. In our previous studies, the pups were weaned at 3 weeks, and the results may reflect this difference. In our previous studies, female offspring were mated at 10 weeks of age, whereas in this study, F1 female mice were mated between 12 and 14 weeks of age. Furthermore, in our previous studies, paper towels stripped into thin strips were fed as nesting material, but in this study, the cotton used in the nest-building assays remained in the cage. In both studies, the mothers were fed nesting materials, but cotton provided greater warmth and softness and made for a

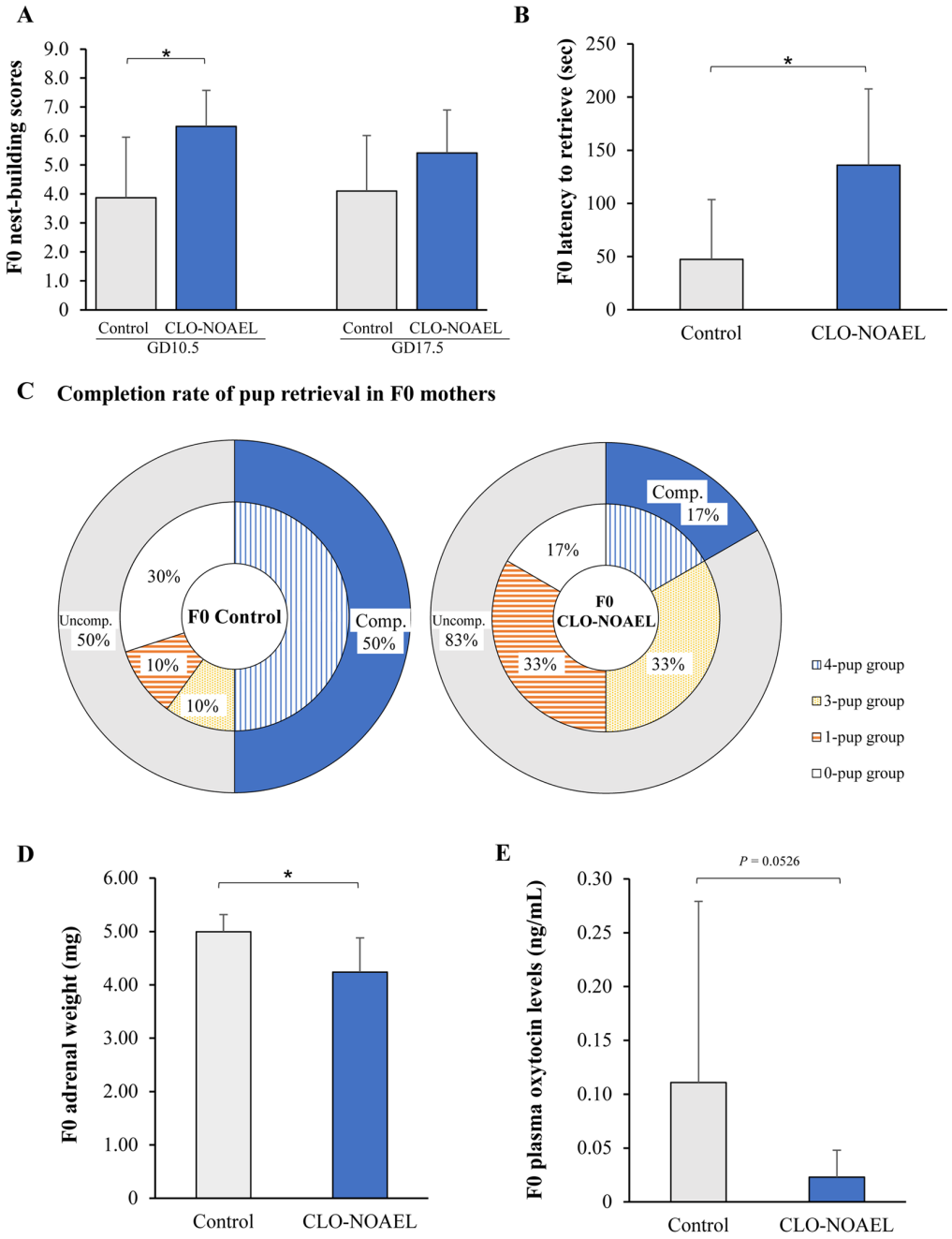

Fig. 2. Behavioral and physiological outcomes in F0 mothers exposed to clothianidin (CLO) at no-observed-adverse-effect-level (NOAEL) doses during pregnancy and lactation. (**A**) Nest-building scores of F0 mothers. Gestational day (GD) 10.5 scores were significantly higher in the CLO-NOAEL group compared to the Control group (P=0.0262). (**B**) In the pup retrieval assessment, latency to retrieve the first pup of F0 mothers. The latency (in seconds) was significantly longer in the CLO-NOAEL group compared to the Control group (P=0.0333). (**C**) Completion rate of pup retrieval in F0 mothers. Mothers who retrieved all 4 pups within 6 min were classified as Completed (Comp.), while those that did not retrieve all pups were classified as Uncompleted (Uncomp.) and are shown in the outer circle. A group of mothers with N pups retrieved is referred to as an "N-pup group", and the number of retrieved pups is shown in the inner circle. Five (50%) of the mothers in the Control group completed retrieval of all pups, compared to 1 (17%) in the CLO-NOAEL group. (**D**) Adrenal weights of F0 mothers. CLO-NOAEL group mothers weighed significantly less than Control group mothers (P=0.0466). (**E**) Plasma oxytocin levels in F0 mothers. The level tended to be lower in the CLO-NOAEL group compared to the Control group (P=0.0526). Values are mean ± SD (n=6–10 mice each). *P<0.05.

more comfortable environment for the mothers around parturition. This difference in nesting material may also have contributed to the differences in results.

This study demonstrated for the first time that NOAEL exposure of CLO in mothers during pregnancy and lactation causes a decrease in plasma oxytocin levels, which in turn causes a decline in maternal behaviors such as pup retrieval. Furthermore, these effects may impair maternal behavior when the offspring mice become mothers of the next generation.

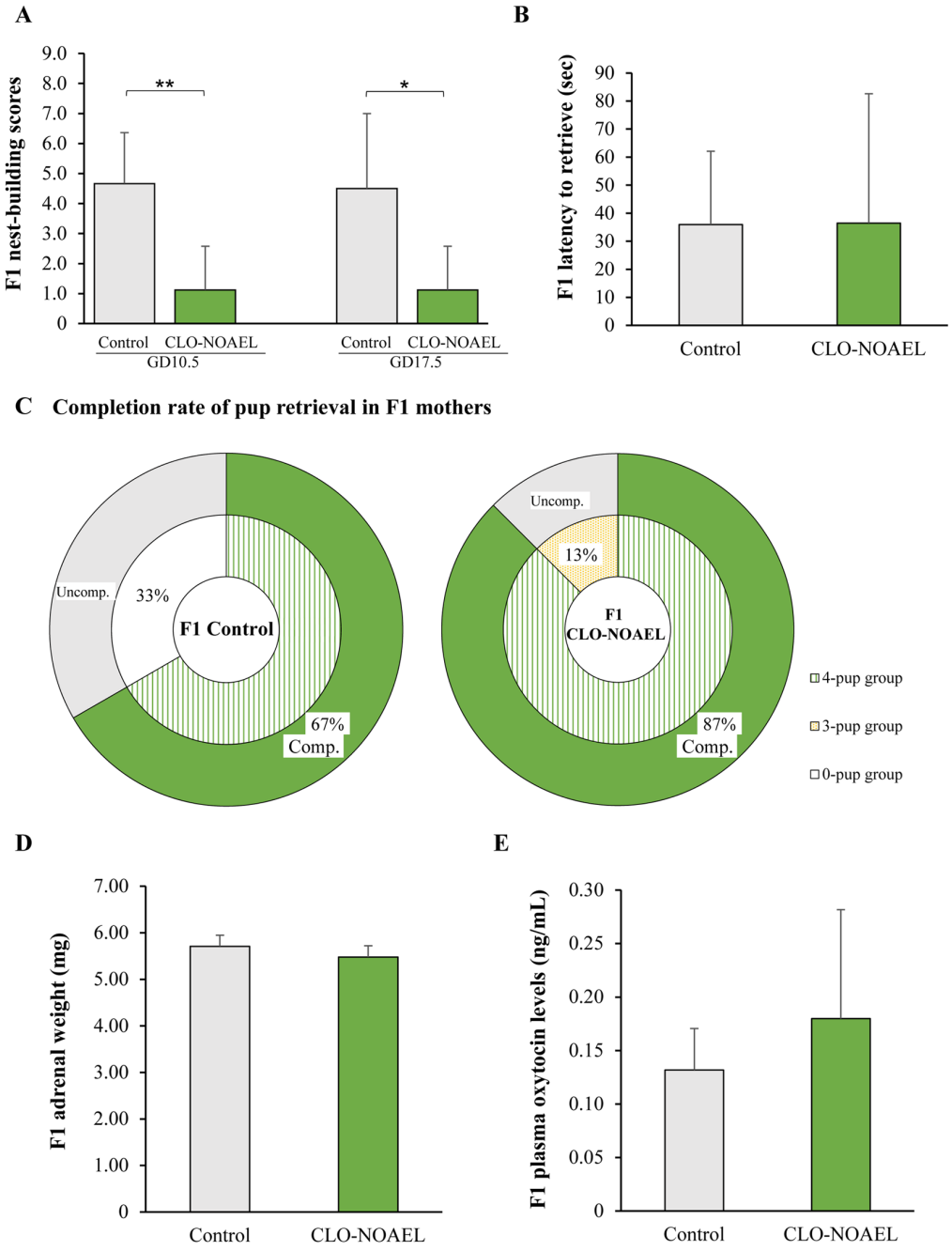

Fig. 3. Behavioral and physiological outcomes in F1 mothers exposed to clothianidin (CLO) at no-observed-adverse-effect-level (NOAEL) doses during fetal and neonatal periods. (**A**) Nest-building scores of F1 mothers. Both gestational day (GD) 10.5 and GD17.5 scores were significantly lower in the CLO-NOAEL group compared to the Control group (GD10.5: P=0.0087, GD17.5: P=0.0236). (**B**) In the pup retrieval assessment, latency to retrieve the first pup of F1 mothers. There was no significant difference in latency between the Control and CLO-NOAEL groups. (**C**) Completion rate of pup retrieval in F1 mothers. Mothers who retrieved all 4 pups within 6 min were classified as Completed (Comp.) while those that did not retrieve all pups were classified as Uncompleted (Uncomp.) and are shown in the outer circle. A group of mothers with N pups retrieved is referred to as an "N-pup group", and the number of retrieved pups is shown in the inner circle. Four (67%) of the mothers in the Control group retrieved all 4 pups within 6 min, compared to 7 (87%) in the CLO-NOAEL group. (**D**) Adrenal weights of F1 mothers. There was no significant difference between the Control and CLO-NOAEL groups. (**E**) Plasma oxytocin levels in F1 mothers. There was no significant difference between the Control and CLO-NOAEL groups. Values are mean ± SD (n=6–8 mice each). *P<0.05, **P<0.01.

POTENTIAL CONFLICTS OF INTEREST. The authors declare that there are no conflicts of interest.

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