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Ethological methods to study the effects of maternal exposure to estrogenic endocrine disrupters A study with methoxychlor

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Abstract

There has been increasing interest, both at the scientific and regulatory level, in the use of ethological methods for evaluating neural effects of endocrine disrupters. We present a series of ethological studies on the effects of maternal exposure to low, environmentally relevant doses (0.02, 0.2, and 2 μg/g mother bw/day) of the estrogenic pesticide methoxychlor (MXC) on behavior. From gestation day 11 to 17, female miee spontaneously drank oil with or without MXC; their maternal behavior was examined from postpartum days 2 to 15. MXC treatment during pregnancy produced slight changes in the expression of maternal behavior: females fed the lower MXC dose spent less time nursing the pups as compared to control dams. Their maternally exposed offspring were subjected to a series of behavioral tests at different ages. Maternal exposure to MXC affected behavioral responses to novelty in both sexes at periadolescence. The onset of male intrasex aggression was delayed in males prenatally exposed to low doses of MXC, since exposed males showed low levels of aggressive interactions during early adoleseence but not after they reaehed adulthood. When adults, MXC-exposed females, but not males showed increased exploration in an unfamiliar open-field. While a sex difference was observed in the control group, with males being significantly more active in the open field than females, prenatal treatment with some MXC doses tended to decrease the sexual dimorphism in activity levels in the novel environment. Ethology, as the evolutionary study of behavior, may provide a framework for integrating a functional perspective (i.e., evolutionary significance) to studies on proximate mechanisms that can account for behavioral alterations induced by developmental exposure to endocrine disrupters.

Keywords: Endocrine disrupters; Maternal behavior; Aggression; House mice; Exploration; Sex differences; Novelty; Development

1. Introduction

A large amount of data indicates that a variety of substances naturally present in the environment or of anthropogenic origin are able to interfere with the functioning of the endocrine system in vertebrates [17,54]. Such interference can range from a disruptive effect to a modulatory effect, depending on the mode of action of the substance, its concentration in the organism and, primarily, the timing of exposure. Over the last years, evidence has been provided that man-made endocrine disrupting chemicals (EDCs) can bind to hormone receptors, such as the intracellular receptors for estradiol and testosterone, and alter development when exposure occurs during critical developmental periods (e.g., fetal life). For example, reproductive system abnormalities in wildlife have been related to endocrine disrupters in fish, alligators and turtles, birds, and mammals [15,16]. EDCs may potentially affect a range of sociosexual behaviors associated with competitive aggression, territorial behavior, mate selection, courtship and mating, parental care, as well as nonsocial behaviors such as exploration (e.g., dispersion patterns, orientation, homing, migration) and homeostatic behaviors (e.g., eating, defensive behaviors). These behaviors are modulated by steroid hormones and determine the individual's fitness, and may thus affect evolutionary processes [44]. Recent studies now confirm that some of the same chemicals implicated in the adverse effects observed in wildlife are also related to altered behavioral responses in animal models (e.g., Refs. [22,41,42,46]) and abnormal

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ncurobchavioral development in humans, including a decrease in IQ [12,30,33,45].

However, the range of outcomes attributable to exposure to environmentally relevant doses (within the range of exposure of wildlife and humans) of EDCs is not well known at all. In particular, functional changes, such as changes in behavioral responses or changes in neural function, as opposed to toxicity or teratology, have not typically been examined in toxicological studies. Behavioral indices may be particularly sensitive to perturbation of hormonal systems because they represent the endpoint of integrated systems, and in particular, behavior is a sensitive and broad indicator of disturbances in the central nervous system. This is a critical issue since toxicological testing for developmental effects has focused on teratology and gross visible damage and not on functional outcomes that could not be detected on gross physical examination [58]. It is well known that gonadal steroids exert potent influences on the nervous system of vertebrates during critical developmental periods and on into adulthood by organizing and reorganizing the neuronal circuitry involved in neuroendocrine and behavioral functions [4,36]. The last 50 years of research on the organizational actions of sex steroid hormones and sexual differentiation suggest that EDCs can potentially have profound influences on behavior. The brain controls behavior and acts as a secondary sex structure, much as the reproductive organs do; steroid hormones secreted by the gonads not only affect the differentiation of accessory and secondary sex structures but also act directly on the brain [17].

There is now clear evidence showing numerous structural and functional differences in the male and female brain, and which further suggests that hormones early in life play a critical role in both the development of these structural sex differences and the sexual differentiation of behavior [23]. For instance, estradiol can induce permanent effects in specific brain regions, in synaptic formation, in dendritic length, in the distribution patterns of serotonergic fibers, in receptor density, and in neuronal connectivity [34,37]. An evolutionary perspective assumes that the male and female neuroendocrine system was shaped by evolutionary processes to maximize fitness. Perturbation of the hormonal milieu during development may interfere with early sexual differentiation processes and alter species-specific, sexually dimorphic behaviors. In this context, strategies employed in ethological analysis can contribute to understanding the impact of estrogenic endocrine disrupters on behavior. Specifically, experiments may be designed to predict whether a behavioral alteration might be adverse based on the perspective of the adaptive significance of the behavior under investigation. This focus on adaptation is different from the traditional experimental approach of behavioral toxicology, which is typically based on proximal causes of behavior and has often used animals as tools to detect alterations in neural or endocrine mechanisms without considering the context and adaptive function of the studied behaviors. An alternative approach is to view behavior from an evolutionary perspective in which behavior, as well as structure and functions of the neuroendocrine system, is considered as part of the organism's adaptive responses to the environment. An important issue of the ethological approach is whether the social and environmental situations in which animals are tested are appropriate for the animal and thus, ecologically relevant [41,42,44]. As Mayr [35] eloquently pointed out, the intertwined but separate issues addressing proximate and evolutionary questions should always be considered in biological research.

In this paper we present a review of our ethological investigations of the effects of maternal exposure to the insecticide methoxyclor (MXC), at concentrations within the range of human exposure and not patently teratogenic, on behavioral responses of male and female house mice (*Mus domesticus*) at different stages of development. MXC is widely used as insecticide on pets, in home gardens, and on crops and livestock. It acts as an agonist of the estrogenic receptor after being metabolized by the liver as shown by in vitro and in vivo studies (see Ref. [18] for a review). Recently, Gray et al. [26] reported a weak antiandrogenic activity of MXC.

We analyzed forms of social and nonsocial behavior sensitive to the action of perinatal gonadal hormones. In particular, the key systems under investigation were those behaviors critical for survival and reproduction (i.e., maternal behavior, aggression and exploration) whose expression is modulated by the neuroendocrine system and that were shaped by evolutionary processes of environmental adaptation to maximize fitness. A second issue was the analysis of developmental trajectories, i.e., testing animals at different ages and for different behavioral endpoints.

2. Maternal treatment and procedures

Female CD-1 mice (*M. domesticus*) were time-mated and group-housed until the start of treatment. In order to reproduce the way animals are exposed to this compound in wildlife, a procedure was developed that allowed oral administration of the chemical to the pregnant female, without disturbing or stressing the animal. This is a critical issue, since handling procedures can be stressful to animals, and stressful events during pregnancy can change the hormonal milieu of the mother and affect neuroendocrine development of the offspring. Before and after time-mating, the females were trained to spontaneously drink a small volume (50 μl) of corn oil purified from tocoferole (Cat #901415, INC, Aurora, OH) from a modified syringe (without the needle and with a larger hole) introduced through the cage top every second day, 2 h after light onset. All females easily learned to drink the oil as soon as the syringe was introduced; this procedure allows accurate administration of chemicals without the stress associated with gavage or injection. On gestation day 11, each female was randomly assigned to one of the

following treatment groups (18–21 females/group): control, MXC 20, MXC 200, or MXC 2000 (0, 20, 200, and 2000 μg of MXC/kg body weight/day, respectively). From gestation days 11 to 17, each female drank 0.1 ml/50 g body weight/day of different solutions of MXC, which was provided to them 2 h after light onset. Within 12 h from parturition (which occurred on gestation day 19), the sex and weight of live newborns was recorded, and each litter was culled to eight pups (three to five males and three to five females) in order to reduce litter size and sex-ratio variability in the growth and development of pups during the postnatal period. At the end of this procedure, the pups were returned to their biological mothers and left undisturbed till weaning. When 26–28 days old, offspring were weaned and mice were group-housed with same-sex littermates (three to six mice/group) until used for testing. All the males and females from each litter underwent the novelty-seeking test after weaning. All the males from each litter were observed for the development of aggressive interactions at 38 and 54 days of age and were then tested for their behavior towards an unfamiliar pup. Two randomly selected males from each prenatally treated litter were tested in the elevated plus maze and then, after 24-h interval, in a resident–intruder test. The remaining two males and two females from each litter were tested in the free-exploratory open-field. The findings on early postnatal development of the offspring resulted from a different experiment [43].

3. Maternal behavior of females fed MXC during pregnancy

When studying the impact of perturbations of early development on behavioral responses it should be considered that behavior is influenced by a large variety of environmental factors affecting both the mother and the offspring, and their interactions, in early and later phases of development. For instance, it is well known that perturbations of the stimuli received by the pups from the mother account for alterations of the neuroendocrine system and behavior as expressed in adulthood [5,25,32,53]. In studies where fetuses or newborns are directly treated or exposed through treatment of the mother to a chemical, not only in toxicological but in pharmacological and endocrinological studies as well, the analyses are generally only addressed to the developing organism, while possible alterations of the mother–offspring interactions are not taken into account nor monitored. Maternal behavior in mammalian species is the result of the complex interaction between the developing offspring and the lactating dam. Slight perturbations of any of the components of the mother–infant interaction may result in alterations of the behavior of the mother and/or of the offspring. In this experiment [39], a detailed behavioral analysis examined the possible effects of MXC on maternal behavior.

The behavior of the lactating females in their home cage was observed by instantaneous sampling during two observation periods of 90 min each on postpartum days 2, 3, 4, 5, 7, 9, 11, and 15. The first observation period (dark period) started 2 h before light onset (at the end of the dark period, when animals are more active), the second 5 h after light onset (light period). During each observation period, the behavior displayed by each dam was checked every 3 min for 90 min (a total of 30 observations). A detailed description of methods can be found in Morellini et al. [39]. Briefly, the following behaviors displayed by the mothers were recorded: nursing, in the nest, licking the pups, nest building, eating, drinking, active behavior around the cage, resting alone, and self-grooming. The scores of the behavioral observations were converted to percentages of the possible maximum [30] of each observational period, and in this form they are presented. The data were transformed to arcsine of the percentages and then analyzed by ANOVA for repeated measures, with one between group factor ("treatment") and two within group factors ("postpartum day" and "light/dark period"). For post hoc analyses, the Duncan test was used.

In general, dams were more active and more involved in self-maintenance behaviors (grooming, eating, and drinking) during the dark period as compared to the light one. Such an increase in activity was associated to a decrease in nest-related behaviors. Moreover, as the litter aged, females spent progressively less time in the nest and nursing, while they increased the amount of time spent eating and drinking and resting alone out of the nest, an index of the start of the weaning process. Relative to the control profile, females exposed to the lowest dose of MXC showed a lower propensity to nurse and stay in the nest and higher amount of eating and resting outside the nest. Such differences in MXC-treated mothers were observed only during the dark period and started on postpartum days 3–7 (Fig. 1). While no differences were observed during the light period, MXC-treated dams showed different behavioral responses relative to control dams during the dark period, when this species is generally more active. As shown in Fig. 1, during the dark period the MXC 20-treated dams spent, over the eight observational days, lower amount of time spent inside the nest ($P=.011$) and nursing ($P=.013$), and more time eating ($P=.005$) and resting outside the nest ($P=.016$) (Duncan test), as compared to the control group. The time-related changes in maternal behavior were analyzed by within-group post hoc analysis on data from single days during the dark period. As compared to postpartum day 2, females of the control group decreased the time spent in nest ($P=.0317$) and nursing ($P=.012$) from postpartum day 11 on, while at postpartum day 15 they increased the time spent eating ($P=.001$) and resting ($P<.001$). Females of the MXC 20 group started to show these changes on their behavioral profile at postpartum day 4 for in nest ($P=.017$) and for nursing ($P=.025$), day 5 for eating ($P=.026$), day 7 for resting ($P<.001$). As shown in Fig. 1, dams of MXC 200 and MXC 2000 groups showed a similar trend, although less pronounced.

These data suggest that the natural decline in nest-related behaviors and the concomitant increase in eating and resting

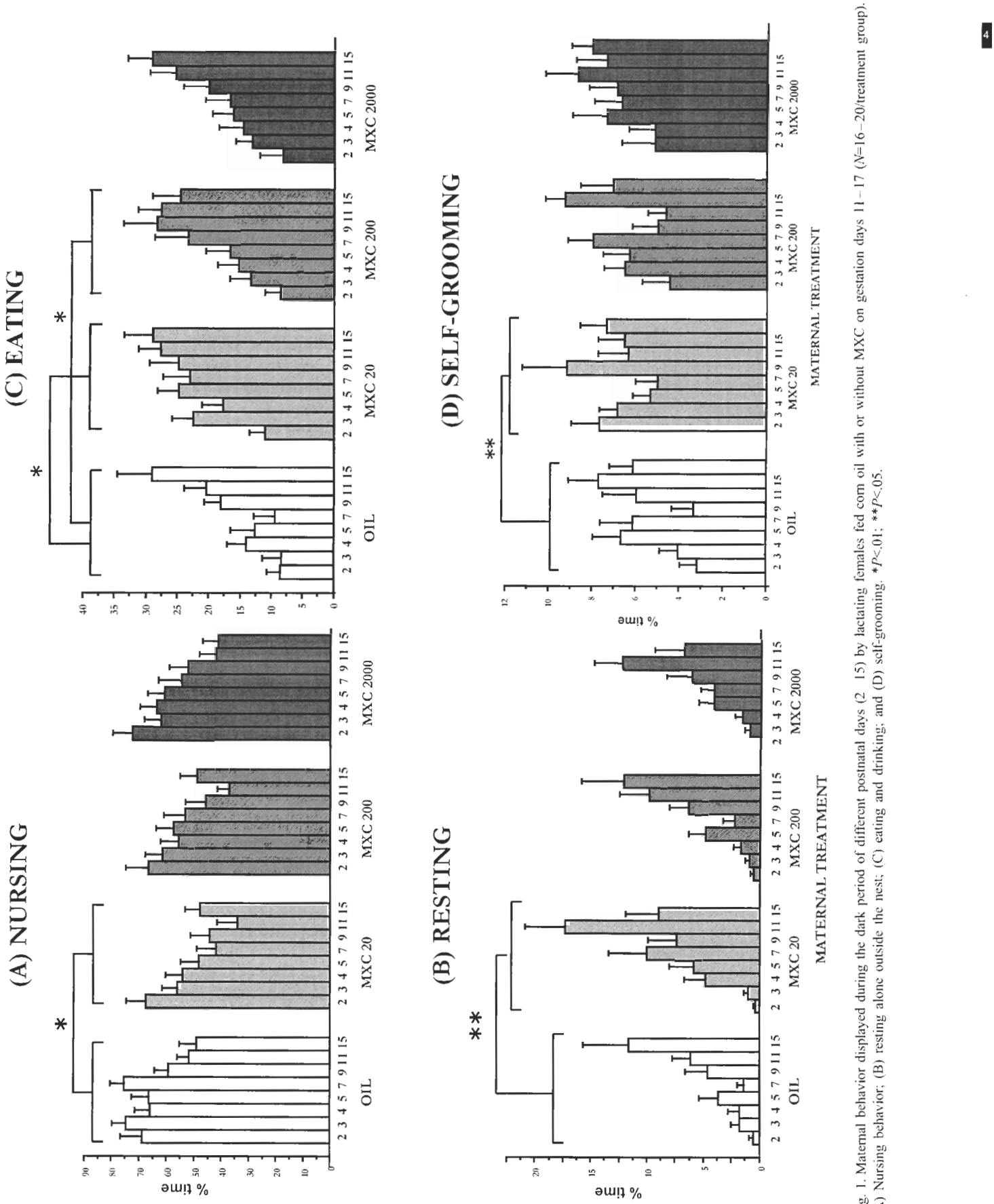

Fig. 1. Maternal behavior displayed during the dark period of different postnatal days (2 15) by lactating females fed corn oil with or without MXC on gestation days 11–17 (N=16–20/treatment group). (A) Nursing behavior; (B) resting alone outside the nest; (C) eating and drinking; and (D) self-grooming. *P<.01; **P<.05.

observed in all dams as offspring aged, started earlier in the MXC-treated dams relative to the control dams. However, the total amount of maternal care received by the pups did not differ between different treatment groups [39], suggesting that this is not a disruption of "normal" maternal behavior as a consequence of exposure to an endocrine disrupter, but rather a slight alteration in the regulation of the timing of mother–offspring interactions. Nevertheless, the functional and adaptive value of such behavioral alterations should not be underestimated, as it might result in perturbations of pups' behavioral development and neuroendocrine profile in adulthood. It is well known that differences in the amount and/or quality of maternal care received as well as in the time weaning starts account for differences of the neuroendocrine and behavioral profile (Ref. [24] for a review).

We hypothesized two different mechanisms underlying the observed alterations: (i) direct effects of MXC on the physiology and the neuroendocrine system of the dam, or (ii) an alteration of litter development and behavior, resulting in a different stimulation and maintenance of maternal behavior during lactation. In the former case, a specific alteration of the neuroendocrine substrates underlying maternal behavior does not seem to be a convincing explanation. Indeed, the regulation of maternal behavior regulated by circulating hormones lasts until 24–36 h postpartum [10], and no differences between treatment groups were observed during the first 48 h postpartum. Moreover, if an estrogenic action of MXC was expected, then an increase, rather than a decrease, in nest-related behaviors might have been observed, since high circulating estrogen activates maternal responsiveness [11]. Alternatively, there may be a more unspecific toxic action of MXC on the dams' metabolism and milk production. While this hypothesis fits with the observation of increased time spent eating for MXC-treated females, it does not explain the decreased propensity to nurse the pups shown by these females, since lower milk quality and production has been suggested to be compensated by an increase in nursing time (see Ref. [13] for review). Moreover, there were no alterations of the dams' body weight, during either gestation or lactation. However, possible interactions of MXC with the nervous system and metabolism of the dams cannot be excluded. On the other hand, the fact that at birth pups born from MXC 20-treated females tended to be heavier than pups born from control females [39] might be an index of an advanced developmental state caused by prenatal exposure to MXC, which in turn could have caused an anticipation of the weaning process.

4. Early postnatal development of the prenatally exposed offspring

In a previous study, the effects of exposure to a wide range of MXC doses (20, 200, 2000, 20,000, and 100,000 μg/kg/day) were recorded on a number of developmental measures on postnatal days 1–10 [43]. More specifically, body weights were measured daily with a digital balance accurate to 0.01 g and all pups were examined for two reflexive responses, righting reflex and cliff avoidance, on postnatal days 2, 5, and 10. Righting reflex is the time taken for a pup that has been placed on its back to turn over with all four feet on the ground. This measure provides information concerning motor and vestibular maturation. Animals were tested for a maximum of 120 s. Animals that did not turn over within 120 s were assigned this score. In the cliff drop aversion test, each pup was placed on a table with the forepaws and face over the edge of the table. The time taken for the pup to turn until it was parallel to the edge of the table was recorded. Animals were given a maximum of 120 s to complete the test. If the animal fell from the "cliff," it was caught and assigned the maximum latency of 120 s. Data were examined and corrected for litter-effect and analyzed by ANOVA.

Prenatal exposure to MXC did not affect body weight of pups on any postnatal day.

Litters prenatally exposed to the lowest dose of MXC (20 μg/kg body weight/day) showed changes in reflexive responses relative to the control group on postnatal days 2 and 5 (Fig. 2). Prenatal exposure to MXC affected cliff avoidance reflex on postnatal days 2 and 5 (F=5.03, P<.01 and F=2.49 P<.05, respectively-Fig. 2B). Pups prenatally exposed to 20 μg/kg/day of MXC showed decreased latency to turn and crawl away from the cliff relative to controls on postnatal days 2 and 5 (P=.12 and P<.05, respectively), while pups exposed to MXC 20,000 showed increased latency to respond on Day 2 (P<.01). Righting reflex was affected by maternal treatment only on Day 2 (F=2.29, P<0.7) (Fig. 2A), with pups exposed to the lower MXC dose showing decreased latency to perform the reflex relative to controls (P<.01). There were no effects of MXC on either reflex on postnatal day 10. These findings suggest that 2–5 day old pups prenatally exposed to the same dose of MXC as the one shown to alter maternal behavior (20 μg/kg) showed an increased performance of some reflexive responses, which may indicate an acceleration in physical and motor development as well as sensory function and/or processing. This may support the hypothesis that an altered developmental state of pups differently stimulated the dams' behavior, since maternal behavior is strongly activated and maintained by the stimuli that the dam perceives from the growing pups [8].

5. Effects on behavioral responses of the prenatally exposed offspring

Early ontogeny is considered a markedly plastic and crucial stage in the organization of CNS structures and subsequent behavior. Acknowledgment of the fact that development is the result of the interaction between the organism and its environment has prompted questions about

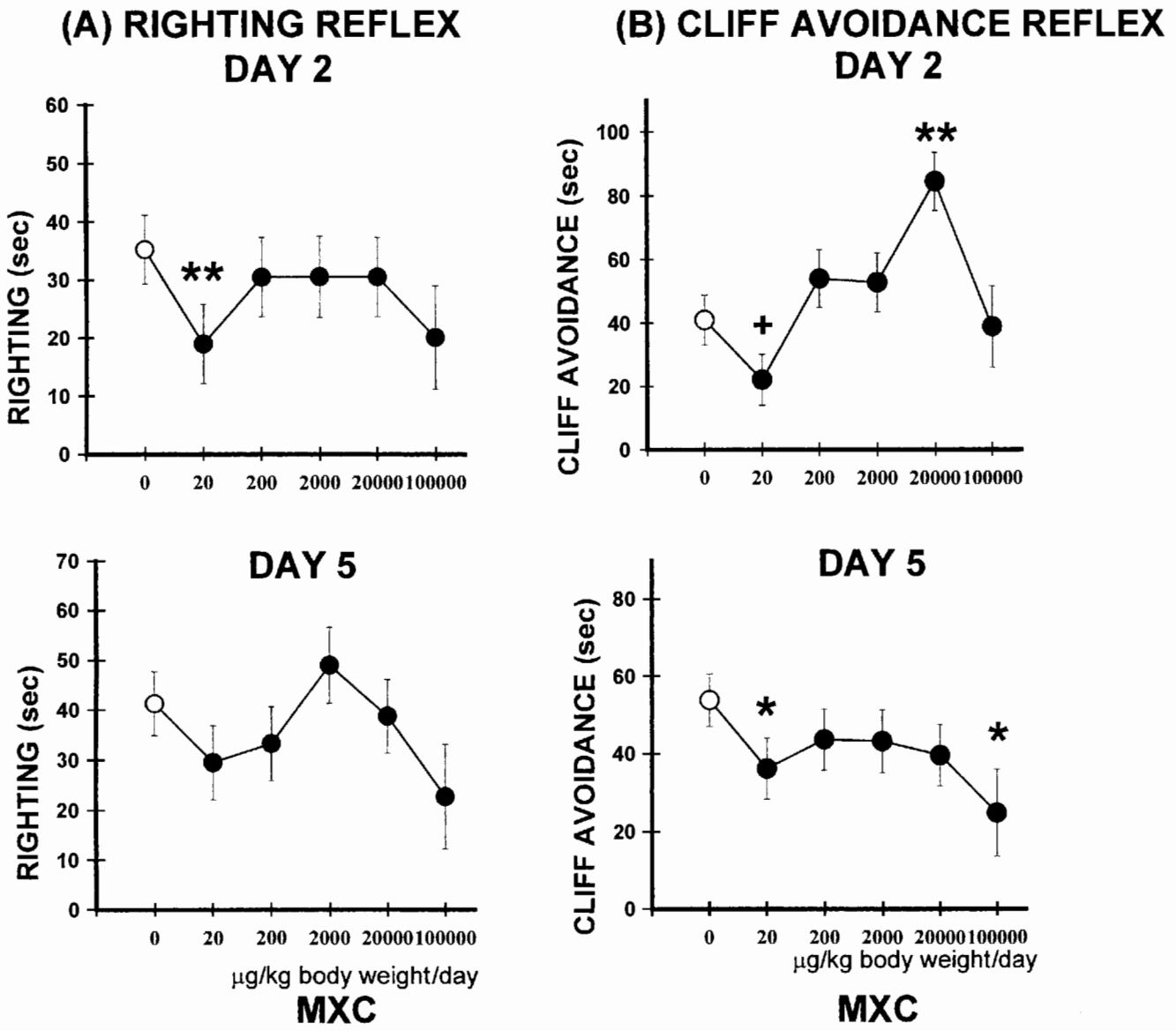

Fig. 2. Mean (±S.E.M.) latency to perform righting reflex (A) and cliff-avoidance reflex (B) on postnatal day 2 or 5 by pups whose mothers were fed MXC during Days 11–17 of pregnancy. *$P<.05$ **$P<.01$ vs. control.

the influence of environmental factors on CNS activity of developing animals. Early events, such as small perturbations of sensory experiences or the hormonal milieu, as well as exposure to psychoactive agents, have been found to alter ontogenetic pathways and to potentially produce huge effects on the central nervous system functioning and behavior later in life (e.g., Refs. [6,52] for review). Exposure to chemicals during early development often inflicts toxic consequences that can be different from consequences inflicted on mature nervous systems. In addition to the modes of damage, however, differences arise in how the damage may be expressed. For example, it may emerge only after a prolonged latency, perhaps in adulthood or as late as senescence.

We report here a series of studies analyzing the effects of prenatal exposure, via maternal treatment, to MXC on forms of social and nonsocial behavior sensitive to the action of perinatal gonadal hormones. The interplay among developmental stage at the time of exposure, age of testing, and response endpoint can be of considerable value in the study of the possible mechanisms of action of estrogenic chemicals on the development of brain and behavior.

5.1. Aggressive behavior and anxiety

Male mice compete among themselves for establishing and holding a territory and achieving dominance. Since reproduction is largely confined to dominant, territorial males, a male's capacity to defeat male conspecifics plays a crucial role in determining reproductive fitness. Male intrasex aggression is also thought to play an important role in spacing conspecifics, thus resulting in the regulation of the density of animals according to ecological conditions

[9]. The development of male aggressive behavior is regulated by genetic, hormonal, and experience factors, and their interactions. The transition between the end of puberty and onset of adolescence, marked by the onset of testicular function, occurs in mice at about 40 days of age. Typically, male littermates at this age begin to show aggressive interactions that later on lead to the establishment of a hierarchical order, with a dominant male, subdominant and/or submissive males [50]. In male mice, prior social experiences can profoundly affect the subsequent responses towards conspecifics [9]. The very first experience of fighting for a male may have long-lasting consequences and significantly alter future behavior. Aggression is not a unitary phenomenon; aggressive competition among males is not limited to male–male agonistic interactions, but includes infanticide as a postmating competition strategy [56]. In these studies we have therefore analyzed the effects of prenatal exposure to MXC on different behavioral responses that are characteristic of the competitive social strategies of male house mice.

We examined the male offspring of females fed MXC during Days 11–17 of pregnancy for (a) the development of aggressive interactions in groups of male littermates during adolescence (39 days of age) and after puberty (54 days of age); (b) behavior towards an unfamiliar pup at about 60 days of age; (c) territorial aggression of adult males (80–85 days of age) in a resident–intruder test; (d) anxiety in response to a novel environment (elevated plus maze test, EPM) [40,41].

At 39 and 54 days of age, same-sex sibling groups of male mice were moved into a novel cage with clean sawdust and were observed for the following 60 min to monitor the occurrence of aggressive interactions between siblings. As shown in Fig. 3A, when males were 39 days old, male siblings prenatally exposed to the MXC 20 μg/kg/day dose showed lower proportion of aggressive interactions as compared to the control group. No differences were recorded in the frequency of aggressive interactions at 54 days of age. However, both when 39 and 54 days old, males prenatally exposed to the MXC 20 μg/kg/day dose showed a significantly longer latencies to display aggressive interactions relative to controls.

As adults (64–66 days old), all males were isolated for 24 h (N=60–66/treatment group) and tested for infanticide behavior towards a 2-day-old unfamiliar pup placed in the male's home cage. Prenatal treatment did not alter the proportion of mice showing infanticide towards an unfamiliar pup (Fig. 3B). However, males prenatally exposed to the lowest dose of MXC (20 μg/kg) attacked the pup with a longer latency as compared to the control group (P<.05).

In a following experiment, male aggressive behavior was examined in adulthood during resident–intruder encounters. The behavior of rodents shown in a resident–intruder paradigm mimics territorial intermale aggression and conforms to what is believed to happen in territorial defense in the field [9]. Therefore, in this experiment, two males from each prenatally treated litter were individually housed for 3 days in a cage, in order to have this become the established home territory of the resident experimental male. After 3 days of isolation, an untreated sexually naive male, matched for age and weight with the resident test animal, was introduced into the cage for 10 min. There were no significant differences between control and prenatally treated males on the proportion of attacking residents, the latency to attack, and the intensity of aggression (Fig. 3C). In a previous study [42], we reported that prenatal exposure to low doses of the synthetic estrogen diethylstilbestrol, and marginally to the estrogenic pesticide o,p'DDT, increased the frequency of territorial aggression in male CD1 mice, an effect that appeared to be related to a decreased latency to attack. However, males prenatally exposed to o,p'DDT displayed a decreased intensity of aggression. Explanations of the effects of prenatal exposure to estrogenic chemicals (DES and pesticides) is complicated by the fact these compounds may not have a linear dose effect but instead typically U curves—acting at several different levels in nervous system; prenatal exposure to different doses of these compounds—or to different compounds—can produce differential effects on a number of behavioral measures. Furthermore, it has been reported that the in vivo metabolite of MXC (bis-hydroxy-methoxychlor) binds with equal affinity to the androgen receptor (and acts as an androgen antagonist) and the estrogen receptor (and acts as an estrogen agonist) in rats [26].

This analysis of male aggressive behavior at different ages and in different context suggests that prenatal exposure to MXC can alter the developmental trajectories of aggression. Specifically, the data show that aggressive behavior of the periadolescent male house mouse was altered after prenatal exposure to the lowest dose of MXC (MXC 20) since around puberty (39 days of age), MXC-exposed male mice showed a decreased frequency of aggressive interactions and a longer latency to attack. With increasing age, this effect was attenuated, but still observed at the age of 54 days. The results relative to the infanticide behavior (at the age of 65 days) seem to parallel those of the aggressive interactions between siblings at 54 days of age, showing a longer time required by males exposed to the lowest dose of MXC (MXC 20) to bite an unfamiliar pup. It is possible that these effects are related either to delayed maturation or to decreased levels of social aggression. In agreement with a delayed maturation hypothesis, the resident intruder tests showed that adult males prenatally exposed to MXC did not significantly differ from unexposed males.

However, the resident–intruder test observations were carried out when subjects were in their home cage (a familiar environment), whereas in the aggressive-interaction tests, males were observed when moved in a novel environment (a clean cage). The emotional response elicited by a novel environment (as in the aggressive interaction paradigms) might have affected the expression of aggression. To evaluate potential effects of prenatal exposure to MXC on emotional responses, the males were tested in the elevated plus-maze

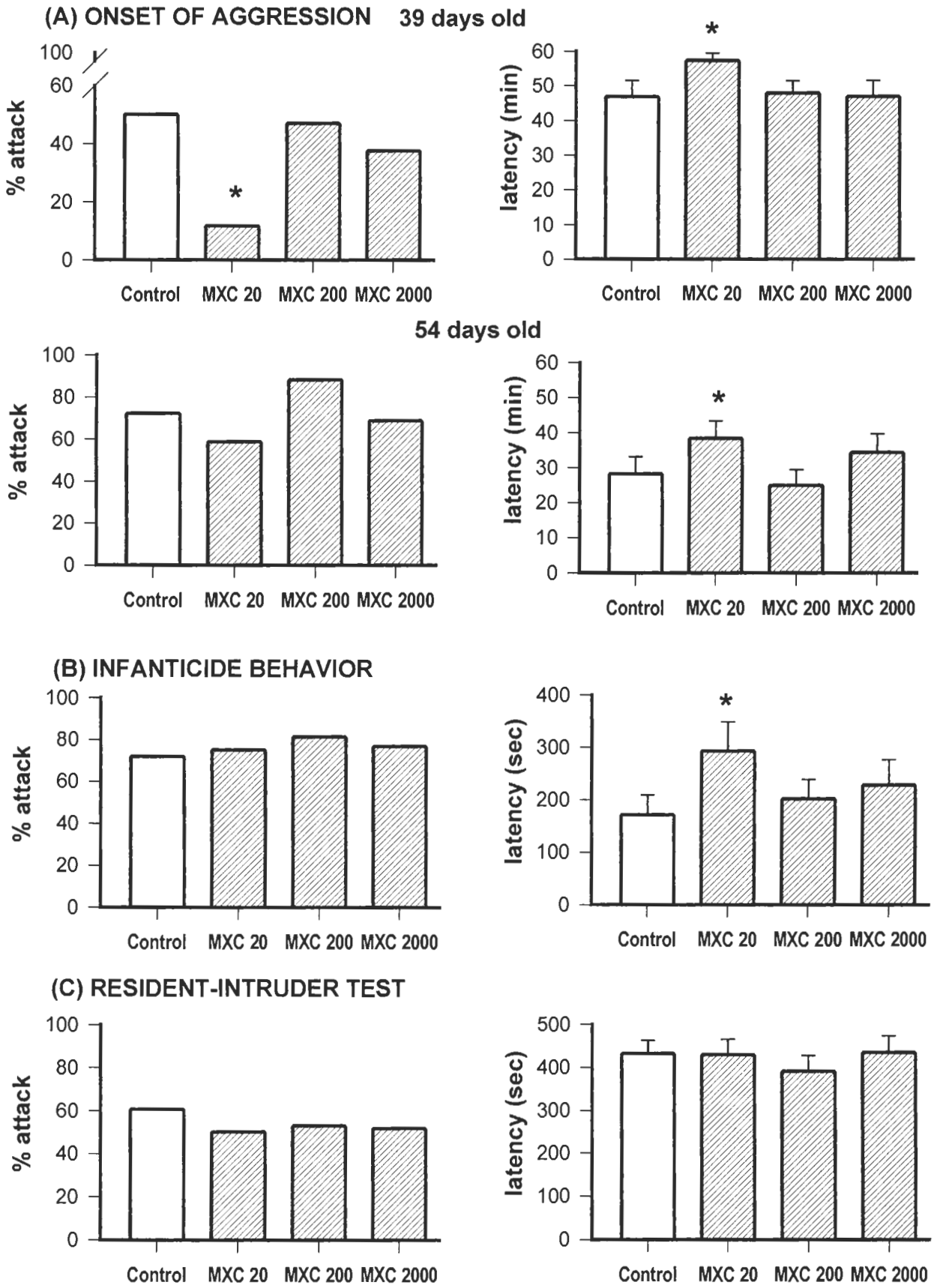

Fig. 3. Percent of mice showing attacks (left column) and mean (±S.E.M.) latency to the first attack (right column). (A) Male sibling groups placed into a novel environment at 39 and 54 days of age; (B) the infanticide test; and (C) the resident–intruder test at about 80 days of age. *$P<.05$ vs. controls.

paradigm, which is one of the most widely used animal model for the study of anxiety [28,47]. In the conventional form of the test, anxiety is routinely assessed by measures of open arm avoidance while locomotor activity is most reliably measured by the frequency of closed arm entries [47]. A series of additional ethological measures, which include stretched attend postures, head-dipping, and grooming, have been linked through factor analysis to risk assessment, directed exploration, and displacement activity, respectively [49]. Farabollini et al. [22] recently reported decreased anxiety in the elevated plus-maze in male rats prenatally exposed to the estrogenic xenobiotic bisphenol A, as indicated by the increased time spent in the open arms and decreased time spent in risk assessment by the exposed rats. In our study [40], males prenatally exposed to MXC 20 and 200 μg/kg/day tended to spend less time in risk assessment (Fig. 4D) but no other anxiety-linked behavior was affected in the elevated plus-maze (Fig. 4A), suggesting that anxiety level was not substantially altered by prenatal exposure to MXC. However, MXC-exposed males showed increased number of entries in arms (Fig. 4C), and spent less time in the central area (Fig. 4B), a profile suggestive of higher locomotor activity that was more marked in the MXC 200 μg/kg subjects (Fig. 4). The finding of increased locomotor activity during the first minutes of exposure to a novel environment such as the elevated plus-maze may be consistent with the higher reactivity to a novel environment observed in periadolescent mice prenatally exposed to MXC described in a previous work [41] and reviewed in the following section. Furthermore, an increased reactivity to novel environments could also explain the previously observed increase in urine marking behavior [57] observed in male mice prenatally exposed to low doses of MXC. Based also on these findings, a possible delay in maturation, rather than increased anxiety, may be responsible for the decreased aggressive behavior recorded in adolescent males prenatally exposed to the lower MXC dose.

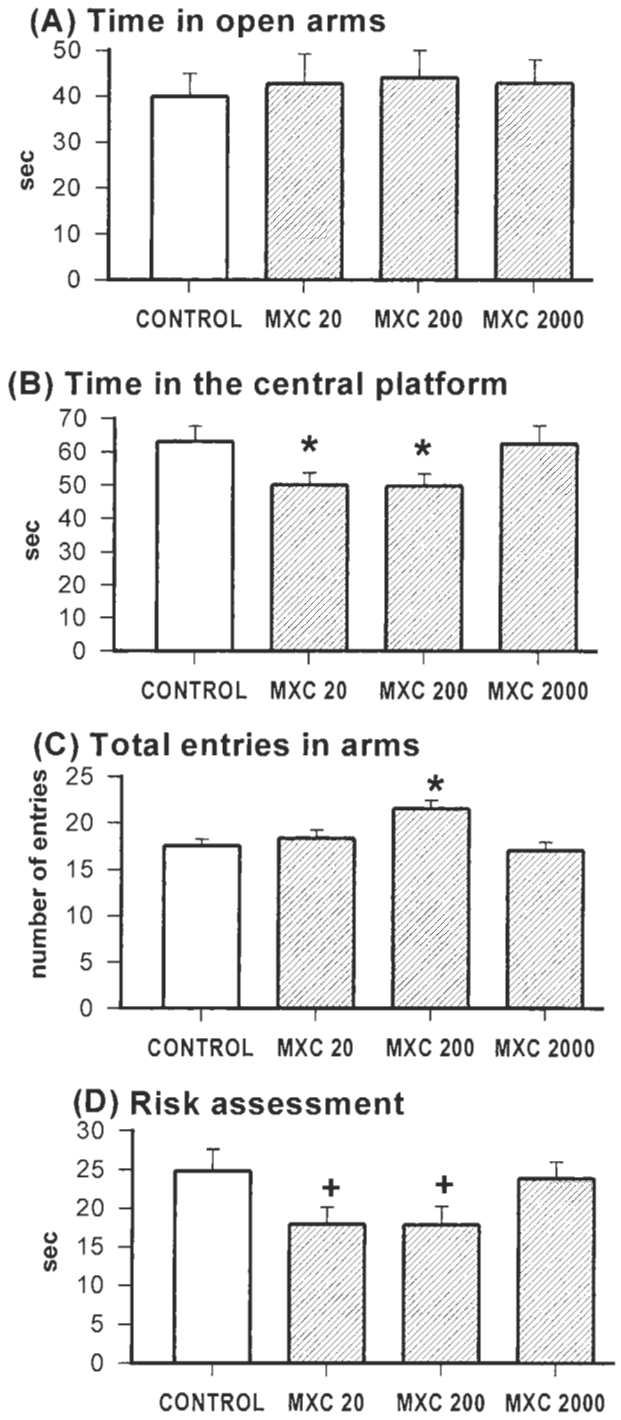

Fig. 4. Exploration in the EPM by males prenatally exposed to different MXC doses. (A) Mean (±S.E.M.) of time spent in open arms; (B) time spent in central platform; (C) time spent in risk assessment; and (D) total entries (frequency) in open and closed arms. *$P<.01$; $^{\backprime}P<.1$.

5.2. Exploration and novelty seeking

In this study we examined the offspring of females exposed to MXC during Days 11–17 of pregnancy for exploration in response to a novel environment. Male and female mice were examined in two experimental paradigms, namely a novelty-seeking test at periadolescence and a modified free-exploratory open field as adults, which can measure the propensity of exploration (curiosity) and preference to novelty, locomotion, and anxiety. In order to measure exploration propensity, we used free-exploratory paradigms, where the animals have the opportunity to choose between a novel and a familiar compartment [27]. The behavior of animals exposed to novel situation results from a competition between an exploratory tendency (novelty seeking, curiosity) and a withdrawal tendency (fear). Generally, animals pay more attention to novel information than to a familiar cues, and they seem to be both attracted and activated by novel stimuli as well as by variations in the set or the intensity of familiar ones [29]. Such enhanced response to environmental changes has adaptive value in that novel stimuli may pose a potential threat or, alternatively, become a possible resource. By using a free-choice paradigm rather than a traditional open field, it is possible to

measure not only specific defensive reactions of mice (such as risk assessment), which have been shown to be a reliable index of anxiety in other fear/defense paradigms [48], but also their exploratory propensity (i.e., novelty seeking or "curiosity") [27].

Early ontogeny is considered a markedly plastic and crucial stage in the organization and regulation of future behavioral responses. In particular, weaning is an important developmental turnpoint, when mice begin to explore the surrounding environment by themselves. Elevated levels of novelty seeking are expressed by mice during periadolescence (reviewed in Ref. [31]), which has been defined as the ontogenetic period that encompasses the 10 days preceding the completion of the puberty period (around postnatal days 30–40) and the first days thereafter. To evaluate response to novelty at weaning (26–27 days of age), the animals were tested following a 24-h period of confinement in the familiar compartment of a free choice novelty preference apparatus (see Ref. [41] for detailed methods). When, on testing day 1, the mice were allowed to freely move from the familiar (Compartment A) to a novel environment (Compartment B), subjects prenatally exposed to MXC were more prompt to explore the novel side of the apparatus (F=2.73, P<.05) (Fig. 5A). Following this test, animals were reconfined in the familiar side (Compartment A) and after 24 h (on testing day 2), they were tested for their response to a novel object placed in Compartment B. As shown in Fig. 5C and D, MXC-exposed mice were more prompt to explore the novel object (F=3.2, P<.03 for the latency to enter the novel object; F=2.08, P<.05 for latency to climb the object). Male and female offspring did not significantly differ in either test and no significant interactions between sex and prenatal treatment were found. However, as shown in Fig. 5A, MXC effects on the propensity to explore the novel side of the apparatus were more pronounced in females. Based on the observation that no difference was recorded in the latency to enter Compartment B on testing day 2 (i.e., mice had had the opportunity to explore Compartment B for 20 min on day 1—see Fig. 5B), these data suggest a specific increase of novelty preference in MXC-exposed mice, rather than a more general effect on activity.

To examine exploratory behavior in adulthood, we used a free-exploratory apparatus as described in detail in Ref. [38]. Briefly, it consisted of two sections: a home cage

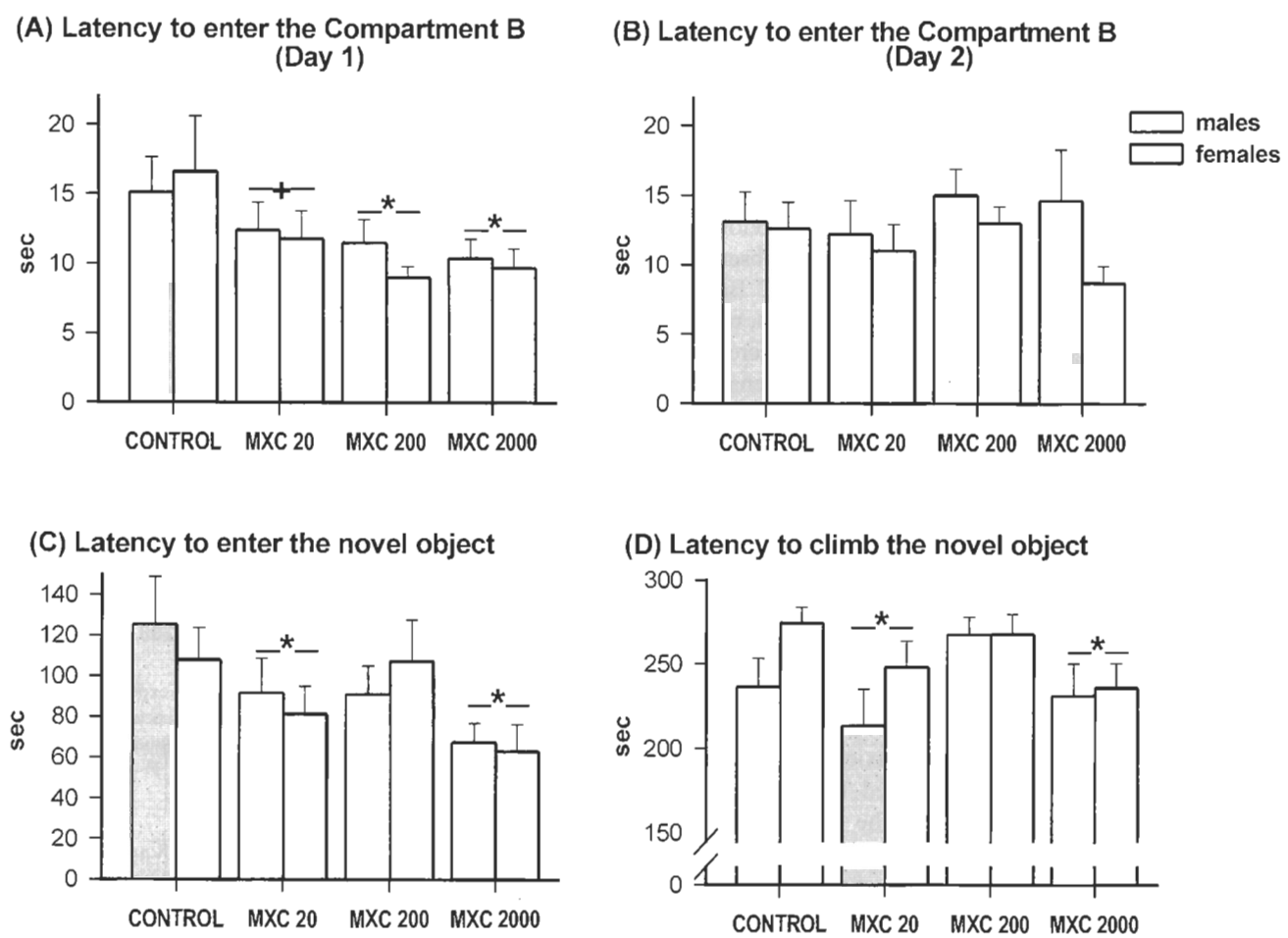

Fig. 5. Mean (±S.E.M.) latency to enter a novel area (A), latency to enter in the area on Day 2 (B), latency to enter a novel object (C), and latency to climb on a novel object (D) in male and female mice prenatally exposed to different doses of MXC. *P<.05, ^{1}P<.08 vs. controls.

and an unfamiliar area (an open-field—OF). Two males and two females per litter (70–80 days old) were individually housed in the home cage section and after 24 h, the removable barrier was removed allowing entrance in the OF. A cutoff of 10 min was used for those animals that did not enter the OF; starting from the first entrance in the OF, behavioral observation lasted 5 min. Data were analysed by 2-factor ANOVA (sex and prenatal treatment). A sex difference was recorded in behavioral responses to the novel arena. In the control group, females entered the arena with longer latencies, showed more risk assessment, spent lower time exploring, and showed lower locomotor activity inside the OF relative to males [38]; females also showed lower levels of rearing, a behavior that is correlated to exploratory activity and novelty seeking. As compared to males, the profile of the control females is suggestive of lower propensity to explore a novel environment and lower activity. Such sex differences in exploratory activity were significantly reduced in the group prenatally exposed to the MXC, in particular in the lower dose group (Fig. 6). Specifically, females prenatally exposed to MXC 20 and 2000 μg/kg displayed higher locomotor activity and rearing (Fig. 6B,D), and spent more time exploring the OF than control females (Fig. 6A) [38]. No effect was observed on males' behavior.

In the light of the evolutionary theory of sexual selection [19], males (the sex with lower parental investment) are expected to be higher than females (the sex with higher parental investment) on behavioral approach systems (including novelty seeking, neophilia, exploratory behavior, risk taking, boldness, sensitivity to reward, and impulsivity) and competition-related behaviors (aggression, territory defense, courtship). This trait can also be associated with the fact that in most mammalian species, natal dispersal is sexually dimorphic, with males that reach puberty usually emigrating from their natal areas whereas females are typically philopatric [21]. Behavioral differences between males and females, as modulated by gonadal hormones during development, determine different social roles and attitudes of males and females. Slight changes in these processes may have long-term consequences on population dispersion and social dynamics.

For additional relevance of this kind of studies, there is increasing evidence in the clinical literature and in experimental studies concerning the association of high levels of sensation/novelty seeking with the expression of risky behaviors and individual vulnerability to drugs of abuse, particularly during adolescence [3,61]. The search of novel stimuli and sensation shares common neurobiological sub-

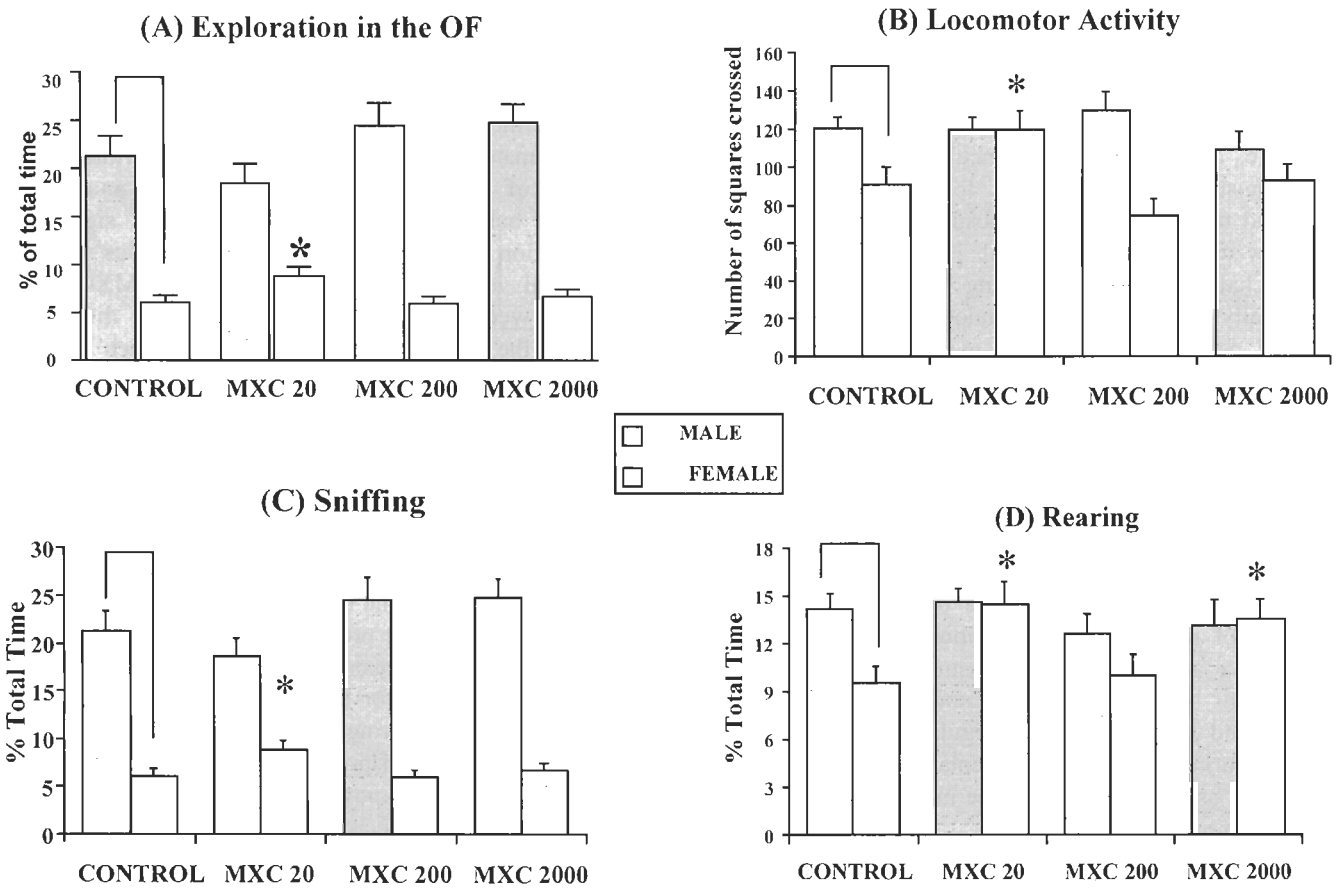

Fig. 6. Exploratory behavior in the open area of the modified free-exploratory paradigm in male and female mice prenatally exposed to different doses of MXC. (A) Time spent exploring the open field; (B) locomotor activity; (C) sniffing (vertical exploration); (D) rearing behavior. *$P<.05$, $^{+}P<.1$ vs. control.

strates with psychostimulants (the reward-related brain mesolimbic dopaminergic pathways) [3]. Our data indicate that individuals exposed to very low doses of an estrogenic compound, and with differing sensitivity to these hormones as a function of sex and age of subjects, may exhibit increased responsiveness to environmental challenges. Further studies are necessary to clarify possible neuroendocrine mechanisms underlying these effects and their possible implications.

To this end, we recently conducted a study to examine possible changes in the dopaminergic systems in the nigrostriatal area of the brain in male and female mice prenatally exposed to the same MXC doses used in the behavioral studies described here. It is recognized that the mesolimbic and nigrostriatal dopaminergic systems represent major structures of the CNS underlying hyperactivity, novelty-induced behavior, reward learning, and attention deficits [2,7,14]. The dopamine system has been reported to be affected by in utero and/or lactational exposure to endocrine disrupters (e.g., Ref. [51]). Preliminary findings suggest a decrease in D1-like receptor density in the nucleus accumbens and olfactory tubercle of adult female mice prenatally exposed to the estrogenic pesticide MXC, at the same doses that caused an increase in novelty-induced locomotor activity [38].

6. Concluding remarks

Ethology, as the evolutionary study of behavior, may provide a framework for integrating a functional perspective (i.e., evolutionary significance) to studies on proximate mechanisms that can account for behavioral alterations induced by developmental exposure to endocrine disrupters. As stated by Colborn et al. [15] "functional changes pose challenges in documenting the extent of a lesion, especially in the case of neuroendocrinological damage." A central nervous system deficit may become evident only upon a specific kind of behavioral challenge and consequences of exposure to endocrine disrupters can be subtle; examination of both learned and unlearned (reflex and phylogenetically specialized) behaviors may reveal subtle deficits in CNS function, which may or may not be accompanied by demonstrable tissue pathology. An ethological approach, which incorporates both proximate and evolutionary explanations in the development of animal models of neuroendocrine alterations, may allow careful qualitative and quantitative assessment of neurobehavioral alterations induced by EDCs and may help to better understand the impact of dysregulation of the hormonal milieu during critical developmental periods on brain and behavior, as well as to clarify the mechanisms of action of these compounds.

In 1872, Charles Darwin [20] laid the conceptual foundation for viewing the behavior of other species as essential evolutionary precursors to human reactions. However, to understand homologies or analogies between animals and humans by means of appropriate animal models, we should consider the context in which an animal is placed and the adaptive/functional significance of that behavior in a particular context. In this perspective, to make appropriate analogies between animal and human behaviors, it is indeed important to consider the function and context of behavior in the model in an evolutionary perspective. Animal models may contribute to elucidating the impact of endocrine disrupters on brain development and behavior, but they imply consideration of the natural life and biology of the animal species studied and of their "natural" behavior. When designing laboratory experiments, the species used, gender, age, experience, environmental features, and their interactions, are all aspects to evaluate in order to recreate the context and hence, the function of the particular behavioral patterns under examination (see also Refs. [1,44]). For instance, developmental effects of toxicants are sometimes misinterpreted to refer exclusively to direct and specific damage to the developing nervous system, while they may depend, at least partially, on alterations to delicate reciprocal mother–pup relationships. An analysis of maternal behavior is therefore important when assessing the effects of chemicals administered via maternal treatment.

As the results of these ethological studies indicate, exposure through a nonstressful administration procedure (i.e., females spontaneously drink corn oil in which the compound is dissolved) of pregnant female mice to low, environmentally relevant (i.e., levels that could be encountered by wildlife and humans in the environment) doses of an endocrine disrupter produced subtle alterations in subsequent maternal behavior and in the behavioral development of their offspring. The detailed analysis of maternal behavior has shown a slight, but significant, perturbation of maternal behavior of females that had consumed very low doses of the pesticide MXC during the last week of gestation. Although it is difficult to evaluate the significance and intensity of such perturbations, it is well known that both variations in maternal care and the response of pups to maternal cues can be responsible for variations in the rate of maturation, such as differences in growth rate or neurobehavioral responses. The effects on offspring of administration of chemicals to pregnant females can thus be amplified due to an interaction of effects on the mother as well as effects on the offspring [24,53]. Variations in the nature of maternal care and in the timing of the weaning process can have important consequences for the subsequent behavioral development of the offspring, as well as for the later reproductive success of both mother and offspring.

The analysis of offspring behavioral development has shown that prenatal exposure to low MXC doses affected early development of neuromotor reflexes, delayed the onset of male aggression, increased the propensity to explore a novel environment in either sex before puberty, and increased novelty-induced locomotor activity only in females in adulthood. More specifically, prenatal treatment

with some MXC doses tended to decrease the sexual dimorphism in activity levels of adult mice in the novel environment. Recent reports [23] suggest that the various components of male versus female differences in brain structures and functions may become expressed during different developmental periods and in response to different hormonal influences. In addition, early ontogeny is considered a markedly plastic and crucial stage in the organization and regulation of future behavioral responses. In particular, weaning and the onset of puberty represent important developmental turnpoints, when mice begin to explore the surrounding environment by themselves and to aggressively interact with conspecifics. Perturbations of the hormonal milieu during fetal development can have a major role in modulating developmental trajectories, which can, in turn, permanently change adult behavior.

In synthesis, we did not detect any "toxic" action of gestational exposure to low doses of MXC, but we did observe slight changes in behavior of lactating females and their offspring at different ages. In other words, the treatment used in this experiment was nontoxic but still active on the organisms with an effect on their behavior. Our understanding of the subtle and complex effects of the alterations in hormonal milieu during fetal life on later behavioral development requires further investigation, involving a wider range of behavioral responses at different developmental stages, and possibly in different species. Although the functional value and quality of such action on the "contaminated" organisms are still to be thoroughly assessed and verified, the peculiar biological characteristics of the EDCs action open new perspectives and questions in neurotoxicological studies. The traditional toxicological paradigm emphasizes a carcinogenic/survival model, a single-compound testing based on the concept of additivity, and the presumption of a threshold dosage below which no adverse effect is evident (the no-observed-adverse-effect level). For a long time, the primary focus of research regarding the effects of man-made chemicals was on their capacity to act as mutagens or to induce gross abnormalities after administration of a dose which has typically been much higher than would be encountered in food, water, or air outside of an industrial workplace. However, with regard to EDCs, the major concern is with functional changes in tissues due to exposure to low, environmentally relevant doses during critical periods in organ development. EDCs are characterized by a delayed response (often measured in years) after exposure to low, physiologically relevant dosages during sensitive periods of organ development in the embryo. The issue of dose in toxicological testing for effects of EDCs is critical. The concept of the threshold dose cannot be applied to EDCs since they mimic or antagonize the actions of endogenous molecules important to development, acting at very low doses, so that the threshold is automatically exceeded with exposure [17,55,58]. Hormones and hormone-mimicking chemicals do not show linear dose–response curves throughout a wide dose range and dose–response relationships are not uniform across all endpoints, ages, genders, and species. Instead, nonmonotonic functions, such as U-shaped or inverted U-shaped curves are commonly found [59]. In fields of science in which receptor-mediated effects are studied (such as responses to neurotransmitters or hormones), it is well recognized that inhibition of response can occur at high doses of a ligand, in part, due to receptor down-regulation. Nonmonotonic dose–response curves are commonly found in endocrinology. For instance, in studies in which pregnant female mice were administered a 5-log range of doses of DES, we obtained a nonmonotonic dose–response function for anogenital distance and body weight at birth, reflex development [43], urine marking rates [57], and adult prostate size in male offspring [59,60]. However, nonmonotonic dose–response curves do not occur for all responses to endocrine disrupters.

The conclusion from our studies, as well as other findings, is that responses to endocrine disrupters cannot be assumed to be monotonic across a wide dose range. These findings suggest that unique outcomes may occur in response to low, environmentally relevant doses of endocrine disrupters that will not be predicted by effects at higher doses. The fact that we have observed effects on behavior at doses of the estrogenic pesticide methoxychlor that are within the range of human exposure and are thus environmentally relevant is of concern since the dose–response methods of studying these same chemicals predict no effect at these low doses.

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