

Sexually Dimorphic Nonreproductive Behaviors as Indicators of Endocrine Disruption

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Measures of cognitive and other behaviors not specifically related to reproduction are often sex-linked. Males and females perform differently on many tasks and often interact with members of their species in dissimilar ways. If such differences are diminished, reversed, or widened by prenatal chemical exposures, a reasonable inference is that exposure interfered with sexual differentiation of the brain, largely, but not exclusively, through interference with the actions of gonadal hormones. Explicit recognition of sex differences in performance is not a prominent feature of toxicity testing, however, except for reproduction studies, and is not a recognized criterion in developmental neurotoxicity testing. In contrast to the low visibility accorded sex differences in testing protocols for the assessment of developmental neurotoxicity, the literature is filled with examples showing that the developing male and female respond differently to many chemical agents, with subsequent expression in behavior. Quite often, even when such differences are reported, further analyses are not carried out nor are subsequent studies conducted for clarification. Moreover, many investigators include only male subjects. Both polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) studies provide several examples of striking differences between the behavioral responses of male and female offspring to developmental exposure. They offer examples, as well, of how to approach the study and analysis of such differences. Given the societal importance of risk assessments applied to potential developmental neurotoxins, studies should be deemed questionable if they fail to include outcome measures based on sexual dimorphisms in nonreproductive behaviors. **Key words:** behavioral toxicology, benchmark dose, brain development, PCBs, rats, schedule-controlled operant behavior, sexual dimorphism, spatial discrimination, TCDD. *Environ Health Perspect* 110(suppl 3):387–391 (2002). <http://ehpnet1.niehs.nih.gov/docs/2002/suppl-3/387-391weiss/abstract.html>

Questions about endocrine disruption and aberrant brain function still seem lodged in two separate toxicological universes. One recent example is a bulletin from the Agency for Toxic Substances and Disease Registry (1). In summarizing its revised assessment of polychlorinated biphenyl (PCB) toxicity, it assigns developmental neurotoxicity to the section titled “Neurodevelopmental Studies.” Under another rubric, the “New Endocrine Disruptor Section,” it points to “. . . the effects of PCBs on breast cancer, estrogenic and antiestrogenic activity, the reproductive system, and thyroid glands.” Apparently the bulletin’s authors failed to connect the neurotoxic properties of PCBs with their endocrine-disrupting properties.

This is hardly the only instance in which the effects of hormones on other organ systems are discussed in isolation from their effects on the brain. Much of the literature on endocrine disruptors has reported on the reproductive system and reproductive performance. Disorders of reproduction in experimental animals represent significant threats to the sustainability of wild populations, of course, and imply potential hazards to human health. The primary hazards to humans, however, appear in less direct guises. In their most subtle and insidious form, they emerge as interference with the course of brain development and resulting

aberrations in behavior. Some of these aberrations appear as alterations of characteristic differences between males and females in nonreproductive behaviors.

Brain development is a tightly orchestrated process. It proceeds through a sequence of stages that continues long after birth. And, pertinent to the theme of this workshop, it is guided by hormones. Gender-specific regional differentiation of the brain and, ultimately, its expression in behavior are guided by the gonadal hormones. The process is delicately balanced, however, and subject to interference by drugs and environmental contaminants.

Although reproductive function is often considered the predominant realm of behavior served by gonadal hormones, appraising the risks posed by environmental endocrine disruptors requires that we ask a much broader question: What are the implications for nonreproductive behaviors? Males and females, both human and otherwise, differ significantly in many aspects of performance. If such differences are diminished, reversed, or widened by perinatal chemical exposures, a reasonable inference is that exposure interfered with the modulatory effects of sex hormones on brain development.

Data bearing on how environmental chemicals influence sex differences in nonreproductive behaviors and their underlying

mechanisms are rather sparse. McGivern and Handa (2) argue forcefully that this question has not been addressed adequately in the drug abuse literature. They note that “[k]nowing the performance of an animal with respect to a nonreproductive behavior does not necessarily predict its behavior potentials for reproductive behaviors.” Their reviews of the cocaine, opiate, marijuana, nicotine, and alcohol literature substantiate their argument. In the case of nicotine, for example, they point to a small set of data indicating that nicotine exposure of the male fetus, at levels seen in pregnant smokers, results in demasculinized behavior patterns in adulthood and note that the implications for more complex behaviors have not been pursued. Such questions warrant extensive exploration because of their influence on public health policy.

Roots of Sex Differences in Behavior

In humans, sex differences in cognitive behaviors are subtle and described primarily by statistical measures. As groups, males and females differ on average in specific cognitive approaches and abilities, although individuals cannot be assigned gender labels based, say, on neuropsychological test performance. As populations, females tend to score better on tests of verbal ability, whereas males as a group tend to score better on tests of spatial ability (3).

Although the basis of such differences has stirred debate, with some critics asserting that their roots are cultural rather than biological (4), the evidence for biological substrates is compelling. Sexual dimorphisms in brain structure in rats include the size of the medial preoptic nucleus and its sexually dimorphic nucleus (SDN-POA) of the hypothalamus, which is considerably

This article is part of the monograph *Impact of Endocrine Disruptors on Brain Development and Behavior*.

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Preparation of this report and the dioxin experiments described in it were supported in part by center grant ES 01247 and research grant ES 08958 from the National Institute of Environmental Health Sciences.

Received 8 January 2002; accepted 4 March 2002.

larger in males (5,6), and the dentate gyrus granule cell layer of the hippocampus, also larger in males (7). In mice, as well, the granule cell layer of the hippocampus is larger in males than in females and is also larger in the right hemisphere than in the left in males but not in females (8). Significant sexual dimorphism in cortical lateralization is also apparent in rodents; male brains tend to exhibit right hemisphere dominance, whereas female brains tend to be more symmetrical (9,10). Sex differences are seen in the bed nucleus of the stria terminalis in rodents, which also is influenced during development by gonadal steroids (11). Human brains also show evidence of sexual dimorphisms; a structure corresponding to the SDN-POA is larger in males (12), but, although presumably involved in male sexual behavior, the connection has yet to be confirmed. Cortical lateralization in humans also may be sex-linked, with indications that, as in rodents, males show more asymmetry (13).

The examples above, drawn from an extensive literature, are meant simply to illustrate that morphological differences between the sexes have been extensively documented. Such differences, although slight in some cases, are clearly relevant to environmental health issues. They may help explain the findings that males display a higher prevalence of mental retardation, learning disabilities, and attention-deficit/hyperactivity disorder than females, that boys recover function less readily from brain damage than girls, and that women, on the other hand, exhibit a higher incidence and prevalence of dementia than men (14).

Because sexual differentiation of the brain is guided primarily by the actions of gonadal hormones but with thyroid hormones involved as well (15), disturbing the appropriate balance of these hormones during development by exogenous agents will produce morphological, neurochemical, and behavioral abnormalities. McEwen (16) succinctly noted that the brain–endocrine axis is in a delicately balanced state during development: “Exogenous mimics can play havoc with brain development and differentiation.”

This delicate balance is forcefully illustrated by the behavioral correlates of the genetic virilizing disease congenital adrenal hyperplasia. Females with this disorder, which exposes them to high levels of androgens during gestation as well as postnatally, exhibit behavioral patterns indicating both masculinization and defeminization (17). For example, as children, they tend to show male preferences for toys; they are more aggressive than normal girls; they show greater spatial abilities; and they are less interested in feminine appearance. Over a

broad range of behaviors, development becomes more prototypically male because of a shift in the balance of gonadal hormones.

McEwen’s statement supports a compelling argument for giving sexually dimorphic behaviors a major role in assessing the developmental neurotoxicity of environmental chemicals, especially those identified, on the basis of other data, as endocrine disruptors. Experiments with PCBs and dioxins (exemplified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]) offer a variety of examples indicating that, during early brain development, males and females are differentially susceptible to their effects, a differentiation that provides important clues about their toxic mechanisms.

Sex Differences in Behavioral Toxicity

Spatial discriminations are frequently cited as among the behaviors distinguishing male and female rodents as well as human males and females (18,19). PCBs and TCDD have been studied with devices and approaches designed to examine their influence on such behaviors when administered perinatally. In one study (20), the *ortho*-substituted congeners PCB 28, PCB 118, and PCB 153 were administered to pregnant rats by gavage on gestational days (GD) 10–16. Neither male nor female offspring of PCB-treated dams displayed differences from controls in performance on a radial arm maze. Testing on a spatial alternation task, implemented on a T-maze and using delays of 0, 15, 25, or 40 sec, revealed slower acquisition by female offspring exposed to one of the three congeners. Males remained unaffected. Because impairment occurred at all delays, the authors interpreted the results to indicate a learning or attentional deficit rather than memory impairment.

The same investigators (21) studied performance on the two tasks after prenatal exposure to TCDD and to the coplanar compounds PCB 77 and PCB 126. Unlike the previous results with *ortho*-substituted PCBs, in this experiment they found decreased errors on radial maze performance, particularly in male offspring, after TCDD administration (totals of 175 and 700 ng/kg administered during GD 10–16), perhaps because the exposed animals adopted a stereotyped behavioral pattern similar to those displayed by rats with nigrostriatal lesions. In a subsequent study (22), male offspring (700 ng/kg on GD 10–16) again showed improved performance on the radial arm maze, but both sexes showed deficits on a reversal learning task, which, unlike the radial arm maze, does not reinforce a stereotyped response strategy. A further exploration of these findings (23) used both the

8-arm maze studied earlier and a 12-arm maze with only eight arms baited and administered two doses, 700 and 1,400 ng/kg, over GD 10–16. This later study again showed enhanced male performance on the 8-arm maze but only at the lower dose. On the 12-arm maze, neither sex differed from controls. The authors suggest that improved performance on the 8-arm maze may have arisen from the stereotyped patterning rather than improved spatial learning or memory.

These discrepant results led the investigators (24) to examine performance on the 12-arm maze in offspring exposed from GD 6 to postnatal day (PND) 21 to the PCB blend Aroclor 1254, whose constituents are composed overwhelmingly of *ortho*-substituted congeners. They baited only eight of the arms with food so that, for efficient performance, the rats had to learn the pattern. Figure 1 shows the number of reference memory errors over the course of the experiment. Working memory errors showed a similar pattern. The figure highlights asymptotic (i.e., steady state) performance and shows contrasting responses to developmental exposure. During this period, in which some sessions were also used for drug challenges, exposed males committed more errors than control males during the no-drug sessions. Treated female rats committed fewer errors than controls. Male–female difference scores were neither calculated nor analyzed, unfortunately. Had they been, the authors might have found more support for their speculation that endocrine disruption underlies these effects.

This complex pattern of results indicates the need to carefully consider the question of experimental design. Multiple doses plus multiple end points, when interactions with sex also are planned as part of the statistical analyses, mean that experimenters must try to provide enough statistical power, by using large enough subject groups, to enable such analyses.

The developmental neurotoxicity of Aroclor 1254 has also been the subject of further studies. Building on the theme of spatial learning, offspring exposed (as above) to 6 mg/kg daily from GD 6 to PND 21 were tested in a situation designed to measure their ability to switch positions between two levers in an operant test chamber (25). They were reinforced with a food pellet for pressing the correct lever. When the correct position switched sides, they then had to relearn the correct location. Exposed male offspring made more errors than controls on the first of five reversals, whereas exposed females made more errors on later reversals; that is, they reached a lower level of asymptotic performance than controls. Although

the authors attempted an analysis of these response patterns, they did not use the kind of mathematically rigorous method needed to quantify serial dependencies. One useful method (26) consists of specifying optimal response sequences, calculating the degree to which the maximizing criterion is met, and plotting the latter over sessions as a measure of acquisition. Another is to use autocorrelation functions to determine serial dependencies (27). The data do reflect, however, sex differences in error patterns in the exposed offspring, explanations which point to endocrine influences.

Sensory Function

Another aspect of sex differences in behavior produced by developmental exposure to Aroclor 1254 appeared in an experiment directed at sensory effects (28). Because of other data suggesting PCB effects on the visual system (29), which, incidentally, revealed sex differences, rats exposed to 1 or 6 mg/kg from GD 6 to PND 21 were trained to perform a signal detection task for the measurement of both absolute and relative brightness thresholds. Part of the training involved an autoshaping procedure designed to train the subjects to press the lever in the chamber for food. Once the rat learned to retrieve automatically delivered food pellets, the next step associated the lever with food peller delivery. Figure 2 shows striking differences between males and females during this period. Control female offspring emitted fewer responses than males, but males and females differed in how exposure affected them. Exposed females responded at higher rates than controls, whereas exactly the opposite pattern appeared in the males. Because, as shown in Figure 2, the dose-response function in males was U-shaped, the authors remarked that “[t]he effect in the females was PCB dose dependent, whereas in the males it was not.” This statement fails to acknowledge that, as often noted, U-shaped functions are frequently seen in toxicology (30). Such phenomena are especially common in studies of endocrine function. For example, in studies of prostate enlargement in mice due to fetal exposure to estradiol or diethylstilbestrol, prostate weight first increased and then decreased with dose, resulting in an inverted-U dose-response relationship (31). The study that relied on only a single 6-mg/kg dose of Aroclor 1254, described above (25), may have excluded a useful result for that reason.

The results of this experiment (28) also showed sexually dimorphic effects on sensory function. Aroclor 1254 exposure during development produced a decrease in sensitivity for females compared with their controls. In males, exposure produced an increase in sensitivity. The authors interpreted the results

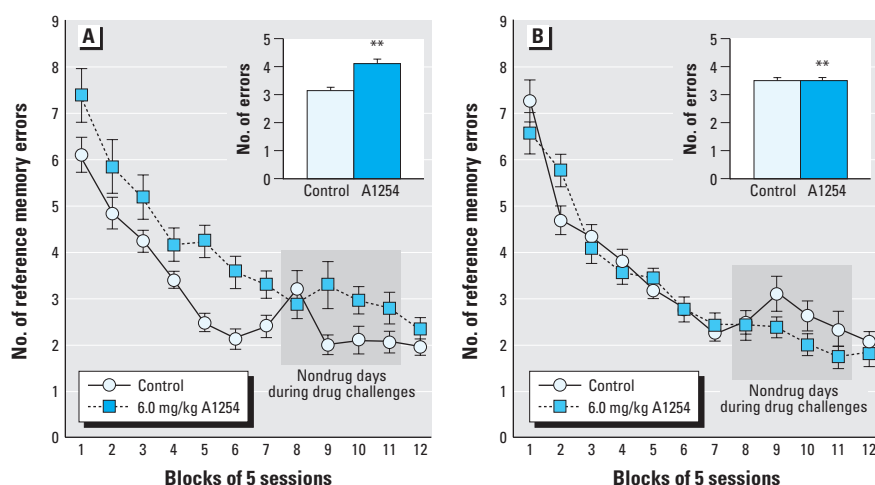


Figure 1. Reference memory errors (mean \pm SE) during performance on a 12-arm radial maze with eight baited arms. The shaded section represents the period during which some trials included drug challenges, but the points shown depict only the nondrug days. Developmental exposure to Aroclor 1254 increased the number of errors made by (A) male offspring but decreased the number made by (B) female offspring. Asterisks (**) denote $p \leq 0.01$. Modified from Roegge et al. (24).

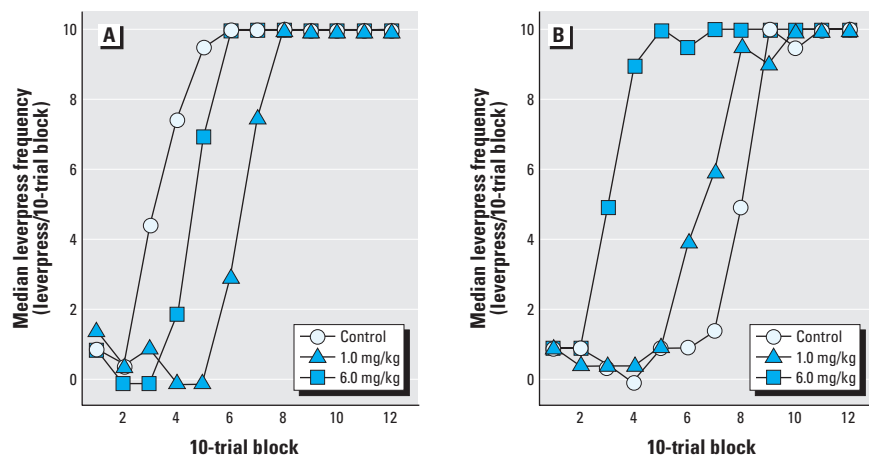


Figure 2. Frequency of lever presses by male (A) and female (B) rats exposed developmentally to Aroclor 1254 during an autoshaping procedure in preparation for visual function testing. Acquisition proceeded faster with exposed females than with controls. In the males, acquisition slowed, and the data show a U-shaped dose-response function. Modified from Geller et al. (28).

of both phases of the behavioral studies as feminization of the males and masculinization of the females, a hypothesis grounded in the superficially paradoxical effects of gonadal hormones administered perinatally. That is, androgens at certain times may feminize male behavior, whereas estrogens may masculinize female behavior. Aroclor 1254 is a complex mixture primarily of *ortho*-substituted PCBs. This property of Aroclor 1254 may explain why, in another study (32), which assayed visual function after developmental exposure to the coplanar PCB 126, no significant effects appeared either on thresholds or on male-female differences.

Schedule-Controlled Operant Performance

Schedule-controlled operant behavior has enjoyed wide applicability in behavioral

pharmacology and toxicology because of its ability to answer questions on how an organism's behavior changes in response to the consequences of the behavior (33). In typical operant situations, normal male rats tend to emit higher overall response rates than females. Ratio schedules, which require a specified number of responses for reinforcement delivery, appear to tax a food-motivated function labeled as "behavioral perseverance" (34). Male rats display food-motivated perseverance across a number of behavioral manipulations. Male rats spend more time than females holding down a lever if holding is food reinforced (35). Male rats are more likely than females to continue to respond on a lever that no longer produces reinforcement (36). Also, under ratio schedules, the performance of castrated males resembles the lower response rates

more typical of control females, suggesting the influence of testosterone (37).

A recent study from our laboratory, based on schedule-controlled operant behavior, evoked distinctly different patterns of responding in rat offspring exposed to TCDD on GD 8 (38). Pregnant dams were given oral doses of 0, 20, 60, and 180 ng/kg TCDD on GD 8. Offspring (one male and one female from each litter) then underwent preliminary lever-press training at 90 days of age for food pellet reinforcement in a two-lever operant chamber. Only one of the levers was active during the experiment. During the first phase of the experiment, the subjects progressed every 4 days through a sequence of fixed ratio (FR) contingencies (1, 6, 11, 21, 31, 41, 51, 61, and 71). That is, they began the series with FR1, requiring only a single lever press to trigger delivery of a food pellet. After 4 days, they advanced to FR6, requiring six lever presses to deliver a food pellet, and so forth until they reached

the final value of FR71, which required 71 lever presses to trigger pellet delivery.

At the completion of the incremental FR phase, we introduced a multiple schedule. FR11 comprised one component. The other component was a differential reinforcement of low rate (DRL) schedule. Here, successive lever presses had to be separated by 10 sec or more to secure reinforcement. The two components alternated and were distinguished by a house light that remained on during the FR segment. Generally, males respond at a higher rate than females on both ratio and DRL schedules, but females typically earn more reinforcements on the latter because they emit fewer premature responses.

Figure 3 shows FR performance during the multiple schedule phase. Exposed males and females moved in opposite directions. Exposed females responded at higher rates than controls; exposed males responded at lower rates. DRL rates moved in the same direction as FR rates. For both components,

the statistical analysis indicated a significant sex-by-treatment interaction. We plotted these interactions by fitting second-order polynomial functions to the difference scores, as shown in Figure 4. The analysis of variance of sex differences documented a significant quadratic trend ($p = 0.01$). The plots show that, although the mean rates for control males exceeded those for control females, the relationship changed across doses. For example, the 60-ng/kg females responded at higher rates than the 60-ng/kg males.

On the basis of these differences, we calculated benchmark doses with the help of the U.S. Environmental Protection Agency's Benchmark Dose (BMD) software. The BMD₁₀ for continuous data is equivalent to an ED₁₀ (10% effective dose) and is used much like an NOAEL (no observed adverse effect level). The 95% lower bound, for example, can be divided by an uncertainty factor such as 100 to provide a reference dose or acceptable daily intake. When we calculated BMD₁₀ values for these data, we found them to be 2.77 ng/kg with a lower 95% lower bound of 1.81 ng/kg for FR rate and 2.97 with a lower bound of 2.02 for DRL rate. Human body burdens of TCDD equivalents were calculated in 1995 to be about 13 ng/kg (39). That is, basing our calculations on male–female difference scores, we see values considerably below those of current human body burdens even without applying a safety or uncertainty factor.

The shape of the dose–response function in this study replicates one we observed in an earlier study (40) that tested only females in an attempt to determine a correlation between estrous cycle phase and the willingness to press a lever for access to a running wheel. Although no correlation emerged, we recorded a similar, significant, U-shaped dose–effect relationship.

Implications

Even this limited survey demonstrates the power of using sexually dimorphic behaviors to illuminate the scope of adverse developmental consequences produced by chemicals with endocrine-disrupting properties. The survey emphasized nonreproductive behaviors because so much of the disruptor literature has focused on reproductive function, perhaps because of its origins in questions about environmental estrogens. The scale of such questions needs to be enlarged substantially to encompass those functions most critical in human life—functions that depend on intact nervous system development. Programs to try to identify potential endocrine disruptors need to include behavioral assays and analyses of the size of sex differences if they are to provide adequate information about health risks.

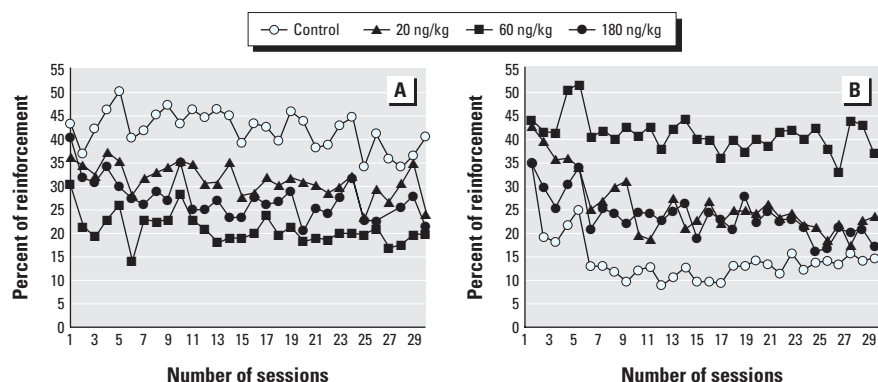


Figure 3. Rate of reinforcement (food pellet delivery) in (A) male and (B) female offspring of rat dams administered TCDD on GD 8. The charts depict performance on the FR component of a multiple reinforcement schedule. TCDD lowered male rates and elevated female rates. Modified from Hojo et al. (38).

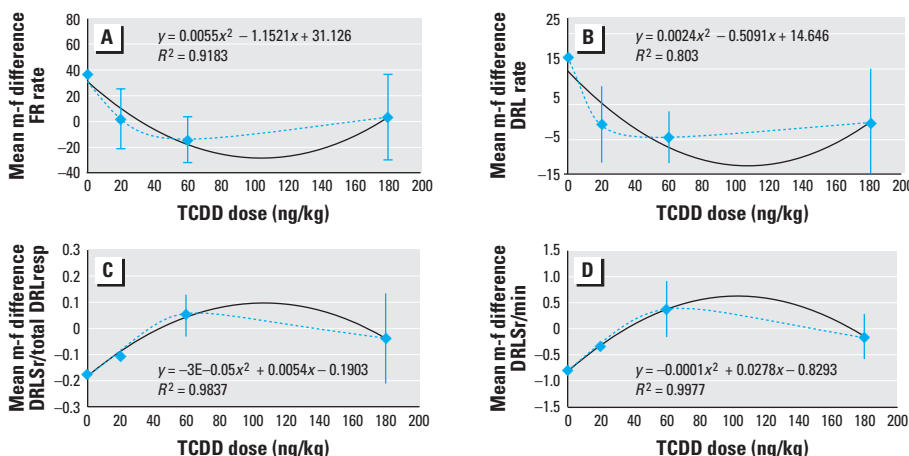


Figure 4. Dose–response functions, based on Hojo et al. (38), plotting male–female difference scores against TCDD dose on GD 8. The dashed line connects the empirical data, and the solid line traces the fitted second-order polynomial. (A) Male–female (m-f) difference for FR response rate (response/minute). (B) Male–female difference for DRL response rate (response/minute). (C) Male–female difference for earned reinforcements (DRLSr) divided by total responses, a measure of response efficiency on DRL. (D) Male–female difference for rate of reinforcement (DRLSr) on the DRL component.

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