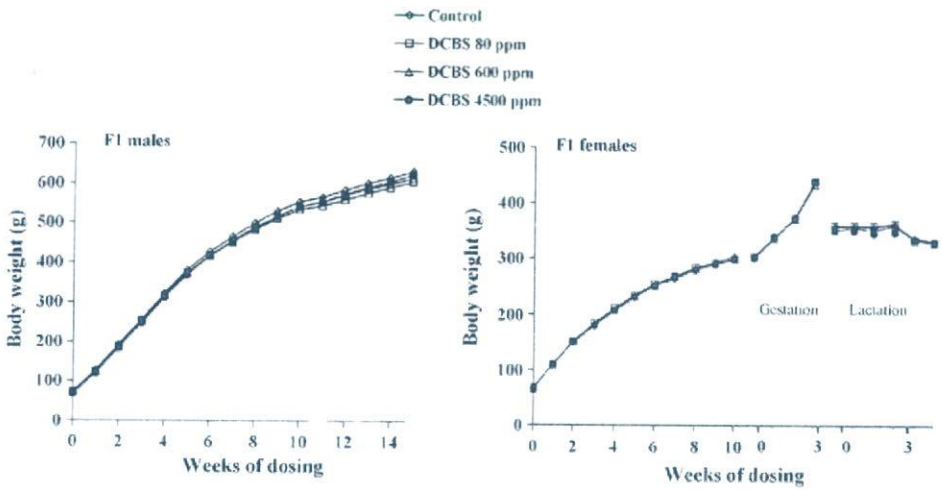

Fig. 2. Body weight of F1 males and females.

The body weights of F0 males and females during dosing are shown in Fig. 1. The body weight and body weight gain of male F0 rats were significantly lowered throughout the dosing period at 4500 ppm. At this dose, the body weight and body weight gain of F0 females were significantly reduced during the first week of dosing and throughout pregnancy and lactation. No compound-related changes in the body weight or body weight gain were noted in F0 males and females at 80 and 600 ppm.

Fig. 2 shows the body weights of F1 males and females during the dosing period. The body weight and body weight gain of F1 males and females exhibited no significant differences between the control and DCBS-treated groups.

There was a significant decrease in food consumption during weeks 1–8 and 13–14 of dosing in F0 males and during the first week of dosing and days 14–21 of lactation in F0 females at 4500 ppm. No significant changes in food consumption were observed in F0 rats of both sexes at 80 and 600 ppm (data not shown).

In F1 male rats, a significant decrease in food consumption was found during weeks 4–7 of dosing at 80 ppm, during week 6 of dosing at 600 ppm and during week 4 of dosing at 4500 ppm. No significant changes were observed in food consumption in F1 females at any dose (data not shown).

The mean daily intakes of DCBS were 5.2, 39 and 291 mg/kg bw in F0 males, 7.2, 54 and 416 mg/kg bw in F0 females, 5.9, 44 and 331 mg/kg bw in F1 males, and 7.4, 55 and 417 mg/kg bw in F1 females for 80, 600 and 4500 ppm, respectively.

3.2. Estrous cyclicity (F0 and F1 females)

Table 1 presents the estrous cyclicity of F0 and F1 females. All F0 females showed normal estrous cycles in all groups, and the length of the estrous cycles was not different between the control and DCBS-treated groups. Although one F1 female each in the control and 600 ppm groups displayed extended diestrous vaginal smears, no significant changes in the incidence of females having normal estrous cycles or length of the estrous cycles were observed.

3.3. Reproductive effects (F0 parents/F1 offspring and F1 parents/F2 offspring)

The reproductive and developmental parameters for F0 parent/F1 offspring are presented in Table 2 . In F0 parent animals, all pairs in all groups copulated, although two females in the con-

Table 1
Estrous cyclicity of F0 and F1 females

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 females				
No. of females examined	24	24	24	24
Females with normal estrous cycles (%)[b]	100	100	100	100
Length of estrous cycles (days)	4.05 ± 0.16[a]	4.01 ± 0.06	4.04 ± 0.15	4.01 ± 0.06
F1 females				
No. of females examined	24	24	24	24
Females with normal estrous cycles (%)[b]	95.8	100	95.8	100
Length of estrous cycles (days)	4.21 ± 0.34	4.05 ± 0.21	4.25 ± 1.08	4.07 ± 0.24

[a] Values are given as the mean ± S.D.
[b] Incidence of females with normal estrous cycles (%) = (no. of females with normal estrous cycles/no. of females examined) × 100.

Table 2
Reproductive and developmental data for F0 parents/F1 offspring and F1 parents/F2 offspring

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 parents/F1 offspring				
No. of pairs	24	24	24	24
Copulation index (%)[b]				
Male/female	100/100	100/100	100/100	100/100
Fertility index (%)[c]	91.7	100	100	100
No. of pregnant females	22	24	24	24
Precoital interval (days)	2.4 ± 1.2[a]	2.8 ± 1.1	2.4 ± 1.0	2.4 ± 1.1
Gestation index (%)[d]	100	100	100	100
Gestation length (days)	22.1 ± 0.4	22.2 ± 0.4	22.0 ± 0.3	22.1 ± 0.3
No. of implantations	13.5 ± 2.1	13.9 ± 1.4	14.6 ± 1.3	13.2 ± 1.5
Delivery index (%)[e]	94.9	94.9	94.3	94.8
No. of pups delivered	12.8 ± 2.1	13.2 ± 1.6	13.8 ± 1.5	12.5 ± 1.7
No. of litters	22	24	24	24
Sex ratio of F1 pups[f]	0.528	0.554	0.506	0.525
Viability index during lactation (%)[g,h,i]				
Day 0	99.0	99.3	99.7	99.0
Day 4	98.7	98.2	96.6	97.6
Day 21	100	99.0	99.5	99.5
Male pup weight during lactation (g)				
Day 0	6.9 ± 0.5	6.7 ± 0.6	6.7 ± 0.6	6.6 ± 0.7
Day 4	11.2 ± 1.1	10.5 ± 1.2	10.5 ± 1.4	10.3 ± 1.0*
Day 7	18.6 ± 1.8	18.1 ± 1.7	17.7 ± 2.5	16.7 ± 1.6**
Day 14	37.2 ± 3.6	36.8 ± 2.4	36.0 ± 4.0	33.6 ± 2.5**
Day 21	62.3 ± 5.6	62.2 ± 3.7	60.2 ± 6.3	55.3 ± 4.8**
Female pup weight during lactation (g)				
Day 0	6.5 ± 0.5	6.3 ± 0.5	6.3 ± 0.5	6.3 ± 0.6
Day 4	10.9 ± 1.3	10.1 ± 1.4	10.0 ± 1.2	9.9 ± 1.0*
Day 7	18.1 ± 1.9	17.1 ± 2.3	17.2 ± 2.3	16.2 ± 1.4**
Day 14	36.3 ± 3.5	34.8 ± 3.6	35.0 ± 4.0	32.8 ± 2.6**
Day 21	60.7 ± 5.2	58.5 ± 6.0	58.2 ± 6.5	53.7 ± 4.5**
F1 parents/F2 offspring				
No. of pairs	24	24	24	24
Copulation index (%)[b]				
Male/female	100/100	100/100	91.7/100	100/100
Fertility index (%)[c]	95.8	91.7	91.7	100
No. of pregnant females	23	22	22	24
Precoital interval (days)	2.7 ± 1.0	2.6 ± 1.4	2.6 ± 1.2	2.8 ± 1.7
Gestation index (%)[d]	100	100	95.5	100
Gestation length (days)	22.3 ± 0.4	22.2 ± 0.4	22.1 ± 0.4	22.1 ± 0.3
No. of implantations	14.1 ± 3.2	13.5 ± 3.7	13.0 ± 4.2	14.3 ± 2.1
Delivery index (%)[e]	90.4	92.9	88.9	91.3
No. of pups delivered	12.7 ± 3.6	12.6 ± 3.7	12.0 ± 4.2	13.0 ± 2.4
No. of litters	23	22	21	24
Sex ratio of F2 pups[f]	0.488	0.516	0.557	0.522
Viability index during lactation (%)[g,h,i]				
Day 0	98.7	99.7	98.3	95.9
Day 4	95.9	94.2	93.1	88.4
Day 21	100[j]	100	97.0	97.7[j]
Male pup weight during lactation (g)				
Day 0	6.8 ± 0.9	6.7 ± 0.8	6.7 ± 0.5	6.7 ± 0.6
Day 4	11.0 ± 2.3	11.1 ± 2.6	10.0 ± 2.1	10.0 ± 1.4[j]
Day 7	18.5 ± 2.7[j]	18.4 ± 3.8	17.1 ± 2.8	15.9 ± 2.3[j,*]
Day 14	37.1 ± 4.0[j]	37.8 ± 6.3	35.5 ± 3.8	32.3 ± 4.1[j,**]
Day 21	62.5 ± 7.0[j]	63.4 ± 9.4	60.6 ± 5.6	53.5 ± 5.9[j,**]

Table 2 (*Continued*)

	DCBS (ppm)			
	0 (control)	80	600	4500
Female pup weight during lactation (g)				
Day 0	6.5 ± 1.0	6.3 ± 0.7	6.3 ± 0.4[k]	6.3 ± 0.7
Day 4	10.5 ± 2.3	10.5 ± 2.5	9.7 ± 2.0[k]	9.5 ± 1.5[l]
Day 7	17.6 ± 2.9[j]	17.7 ± 3.8	16.3 ± 2.8[k]	15.5 ± 2.2[l]
Day 14	35.9 ± 4.1[j]	36.6 ± 5.7	33.5 ± 4.9[k]	31.7 ± 3.9[l,*]
Day 21	59.6 ± 6.6[j]	60.7 ± 8.5	56.3 ± 7.0[k]	52.0 ± 5.7[l,**]

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[f] Sex ratio = total no. of male pups/total no. of pups.
[g] Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.
[i] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 4 after cull) × 100.
[j] Data were obtained from 22 litters because one female that died on day 5 of lactation was excluded from the data.
[k] Data were obtained from 20 litters because one female had no female pups.
[l] Data were obtained from 23 litters because one female that experienced a total litter loss on day 3 of lactation was excluded from the data.
[*] Significantly different from the control, $p < 0.05$.
[**] Significantly different from the control, $p < 0.01$.

trol group did not become pregnant, and all pregnant females in all groups delivered live pups. There were no significant differences in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F1 pups delivered, sex ratio of F1 pups, or viability of F1 pups during lactation between the control and DCBS-treated groups. No malformed F1 pups were found in any groups. A significantly lower body weight was observed in male and female F1 pups at 4500 ppm on PNDs 4, 7, 14 and 21.

The reproductive and developmental parameters for F1 parent/F2 offspring are also shown in Table 2. Two F1 males in the 600 ppm group did not copulate. One female in the control group and two females each in the 80 and 600 ppm groups did not become pregnant. One pregnant female in the 600 ppm group did not deliver. One dam in the control group died on day 5 of lactation, and her pups were euthanized. One dam experienced a total litter loss by PND 3 at 4500 ppm. No significant changes in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F2 pups delivered, sex ratio of F2 pups, or viability of F2 pups during lactation were observed. Oligodactyly in one female of the control group and microphthalmia in one male at 80 ppm were observed. Body weights of F2 pups at 4500 ppm were significantly lowered on PNDs 7, 14 and 21 in males and PNDs 14 and 21 in females.

3.4. Developmental landmarks (F1 and F2)

Physical development of F1 and F2 pups is presented in Table 3. There was no significant difference in the age of male and female F1 and F2 pups that displayed pinna unfolding, or eye opening between the control and DCBS-treated groups. The completion of incisor eruption was delayed in male and female F1 pups at 80 ppm and in male and female F2 pups at 80 and 4500 ppm. The AGD and AGD per cube root of body weight ratio in male and female F1 and F2 pups were not significantly different between the control and DCBS-treated groups.

Reflex ontogeny in F1 and F2 pups is shown in Table 4. All male and female F1 pups in all groups completed the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18. In F1 pups, no significant difference was observed in the response time of the surface righting reflex or the negative geotaxis reflex between the control and DCBS-treated groups. Of the F2 pups, one female did not complete the surface righting reflex and one male did not complete the mid-air righting reflex at 80 ppm, one female did not complete the mid-air righting reflex at 600 ppm, and one female did not complete the negative geotaxis reflex at 4500 ppm; however, no significant difference was found between the control and DCBS-treated groups in the completion ratio and response time for these reflexes.

Table 5 presents data on sexual development in F1 rats. Although a significant delay in the age of preputial separation in males was noted at 4500 ppm, the body weight at the age of preputial separation was not significantly different between the control and DCBS-treated groups. In females, a significantly delayed age of vaginal opening and a higher body weight at the age of vaginal opening were found at 600 and 4500 ppm.

3.5. Behavioral effects (F1)

Spontaneous locomotor activity in 10 min intervals for a total of 60 min was not significantly different between the control and DCBS-treated groups in male and female F1 rats (data not shown).

Fig. 3 shows the results of the water filled T-maze test in F1 males and females. The pre-test swimming trials in the straight channel on the first day of the T-maze test revealed that all F1

Table 3
Physical development in F1 and F2 pups

	DCBS (ppm)			
	0 (control)	80	600	4500
F1 pups				
No. of litters examined	22	23	24	24
Age at pinna unfolding (days)				
Male	2.7 ± 0.5[a]	2.7 ± 0.5	2.9 ± 0.3	2.7 ± 0.5
Female	2.6 ± 0.6	2.6 ± 0.6	2.9 ± 0.4	2.7 ± 0.5
Age at incisor eruption (days)				
Male	10.2 ± 0.6	10.8 ± 0.6**	10.3 ± 0.6	10.5 ± 0.4
Female	10.1 ± 0.6	10.7 ± 0.7**	10.2 ± 0.7	10.2 ± 0.6
Age at eye opening (days)				
Male	14.5 ± 0.6	14.5 ± 0.5	14.7 ± 0.5	14.6 ± 0.5
Female	14.4 ± 0.6	14.5 ± 0.7	14.4 ± 0.4	14.5 ± 0.5
AGD				
Male pup AGD (mm)	5.60 ± 0.28	5.50 ± 0.28	5.51 ± 0.41	5.54 ± 0.28
Male pup AGD/($BW^{1/3}$)	2.51 ± 0.09	2.52 ± 0.08	2.52 ± 0.12	2.55 ± 0.09
Female pup AGD (mm)	3.02 ± 0.11	2.95 ± 0.14	2.99 ± 0.14	2.96 ± 0.14
Female pup AGD/($BW^{1/3}$)	1.36 ± 0.05	1.37 ± 0.06	1.39 ± 0.04	1.38 ± 0.04
F2 pups				
No. of litters examined	23	22	21	23
Age at pinna unfolding (days)				
Male	2.7 ± 0.8	2.7 ± 0.7	2.8 ± 0.6	2.7 ± 0.5
Female	2.7 ± 0.8	2.7 ± 0.8	2.8 ± 0.4[c]	2.6 ± 0.6
Age at incisor eruption (days)				
Male	9.7 ± 0.7[b]	10.6 ± 0.9**	9.9 ± 0.6	10.3 ± 0.8*
Female	9.8 ± 0.7[b]	10.4 ± 0.8*	10.0 ± 0.6[c]	10.4 ± 0.9*
Age at eye opening (days)				
Male	14.4 ± 0.7[b]	14.6 ± 0.8	14.3 ± 0.7	14.6 ± 0.6
Female	14.3 ± 0.6[b]	14.4 ± 0.8	14.4 ± 0.5[c]	14.5 ± 0.7
AGD				
Male pup AGD (mm)	5.54 ± 0.51	5.60 ± 0.55	5.39 ± 0.56	5.47 ± 0.38
Male pup AGD/($BW^{1/3}$)	2.50 ± 0.12	2.53 ± 0.14	2.51 ± 0.12	2.55 ± 0.08
Female pup AGD (mm)	2.93 ± 0.19	2.91 ± 0.22	2.88 ± 0.19[c]	2.85 ± 0.18
Female pup AGD/($BW^{1/3}$)	1.34 ± 0.04	1.34 ± 0.06	1.35 ± 0.03[c]	1.35 ± 0.05

[a] Values are given as the mean ± S.D.
[b] Data were obtained from 22 litters because one dam that died on day 5 of lactation was excluded from the data.
[c] Data were obtained from 20 litters because one female had no female pups.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

rats in each group could swim satisfactorily, and no significant changes in the elapsed time to traverse the straight channel were observed. In males, no significant differences were observed between the control and DCBS-treated groups in the elapsed time and number of errors in on days 2–4 of the T-maze test. In females, a significantly longer elapsed time at 600 and 4500 ppm and more errors at 4500 ppm were noted on day 2 of the T-maze test. There were no significant differences in the elapsed time or number of errors on days 3 and 4 of the T-maze test in female rats between the control and DCBS-treated groups.

3.6. Necropsy and histopathology (F0, F1 and F2)

There were no compound-related gross lesions or microscopic alterations in the reproductive organs of F0 and F1 males and females showing reproductive difficulties. No compound-related gross lesions or remarkable microscopic alterations of tissues and organs, including the reproductive organs, were noted in F0 and F1 males and females in the highest dose group and dead animals before the scheduled terminal sacrifice. In the histopathological examinations of the ovary in F1 females, no significant difference was noted in the number of primordial follicles (mean ± S.D.) between the control (323 ± 57) and 4500 ppm (255 ± 109) groups. There were no compound-related gross lesions or microscopic alterations in male and female F1 and F2 pups, including pups that died before weaning (data not shown).

3.7. Organ weights (F0 adults)

The body weight at the scheduled terminal sacrifice was significantly lowered at 4500 ppm in males and females. Sig-

Table 4
Reflex ontogeny in F1 and F2 pups

	DCBS (ppm)			
	0 (control)	80	600	4500
F1 pups				
No. of pups examined (male/female)	22/22	24/24	24/24	24/24
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Surface righting reflex response time (s)				
Male	2.1 ± 1.6[a]	1.5 ± 0.5	2.4 ± 2.3	1.8 ± 1.2
Female	2.8 ± 3.4	1.6 ± 0.6	1.9 ± 0.9	3.4 ± 3.9
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Negative geotaxis reflex response time (s)				
Male	14.5 ± 8.0	15.4 ± 8.2	13.8 ± 6.4	16.0 ± 7.5
Female	15.3 ± 6.8	14.1 ± 6.0	15.4 ± 6.2	18.3 ± 7.6
Mid-air righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
F2 pups				
No. of pups examined (male/female)	22/22	22/22	21/20	23/23
Surface righting reflex completion rate (%)				
Male/female	100/100	100/95.5	100/100	100/100
Surface righting reflex response time (s)				
Male	2.5 ± 1.6	2.2 ± 1.8	1.7 ± 0.5	2.1 ± 1.9
Female	2.6 ± 1.8	2.4 ± 2.0[b]	2.5 ± 1.7	3.2 ± 4.5
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/95.7
Negative geotaxis reflex response time (s)				
Male	15.3 ± 6.3	17.2 ± 7.4	14.4 ± 5.7	16.1 ± 4.9
Female	16.9 ± 7.2	14.0 ± 6.5	12.6 ± 8.1	16.0 ± 6.2[c]
Mid-air righting reflex completion rate (%)				
Male/female	100/100	95.5/100	100/95.0	100/100

Surface righting reflex on postnatal day 5, negative geotaxis reflex on postnatal day 8 and mid-air righting reflex on postnatal day 18 were examined three times.
Completion rate (%) = (number of animals showing all successful responses of thee trials/number of animals examined) × 100.
[a] Values are given as the mean ± S.D.
[b] Data were obtained from 21 pups.
[c] Data were obtained from 22 pups.

Table 5
Sexual development in F1 males and females

	DCBS (ppm)			
	0 (control)	80	600	4500
Male preputial separation				
No. of males examined	24	24	24	24
Age (days)	41.3 ± 1.6[a]	41.4 ± 1.6	41.8 ± 1.6	42.8 ± 1.5**
Body weight (g)	226.9 ± 20.3	226.5 ± 18.5	228.3 ± 17.0	229.6 ± 17.5
Female vaginal opening				
No. of females examined	24	24	24	24
Age (days)	29.6 ± 1.0	30.0 ± 1.7	31.2 ± 1.7**	31.1 ± 1.3**
Body weight (g)	104.6 ± 9.4	109.1 ± 10.6	112.1 ± 13.8*	112.3 ± 9.1*

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

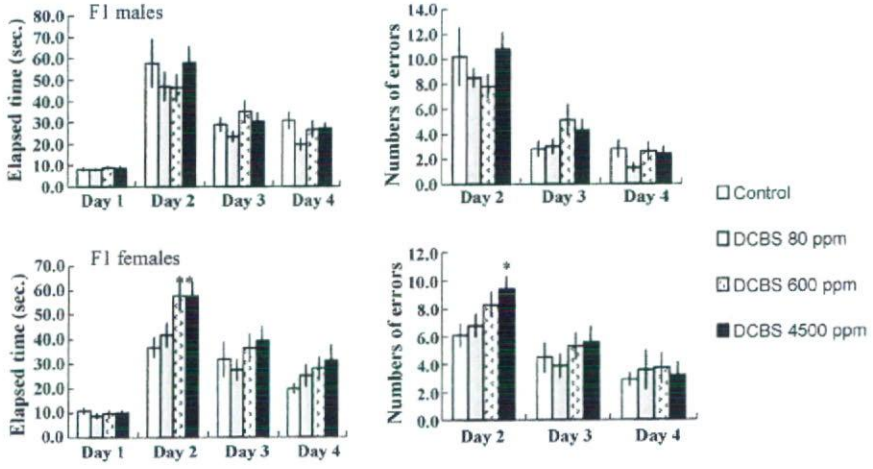

Fig. 3. Performance in water-filled multiple T-maze in F1 males and females. Each rat was allowed to swim in a straight channel on day 1, and then tested in the maze for the next consecutive three days (days 2–4). Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

nificantly decreased absolute weights of the spleen and adrenal gland, and increased relative weights of the brain, thyroid, liver, kidney and testis were detected at 4500 ppm in males. A significant increase in the absolute weights of the brain at 80 and 600 ppm and the pituitary at 80 ppm, and decrease in the relative weight of the spleen at 80 and 600 ppm was observed in F0 females. Significantly decreased absolute weight of the spleen, and increased relative weights of the brain, kidney, and adrenal gland were found at 4500 ppm in females (data not shown).

3.8. Organ weights (F1 weanlings and adults)

The organ weights of male and female F1 weanlings are presented in Table 6. The body weight at the scheduled sacrifice was significantly lowered in males and females at 4500 ppm. The relative weights of the kidney at 80 ppm and the liver at 600 ppm were significantly higher in males. Significant decreases in the absolute weights of the brain, thymus, liver, kidney, adrenal gland, epididymis, and ventral prostate, and decrease in both the absolute and relative weights of the spleen, and increase in the relative weights of the brain, liver and testis were all observed at 4500 ppm in males. A significantly increased relative weight of the kidney at 80 ppm and decreased absolute weight of the ovary at 600 ppm was found in females. The absolute weights of the brain, thymus, liver, kidney, spleen, adrenal, ovary and uterus, and the relative weight of the spleen were significantly lowered at 4500 ppm in females. In this group, significantly higher relative weights of the brain and liver were also observed in females.

Table 7 shows the organ weights of male F1 adults at the scheduled terminal sacrifice. The absolute and relative weights of the thymus were significantly lower at 80 ppm in males. A significantly decreased absolute weight of the brain, decreased absolute and relative weights of the seminal vesicle, increased relative weight of the kidney, and increased absolute and relative weights of the liver were noted at 4500 ppm in males.

The organ weights of female F1 adults at the scheduled terminal sacrifice are shown in Table 8. The absolute weight of the brain at 80 and 600 ppm, and the relative weights of the liver and kidney, and the absolute and relative weights of the adrenal gland at 4500 ppm were significantly increased.

3.9. Organ weights (F2 weanlings)

Table 9 presents the organ weights of male F2 weanlings. The body weight at sacrifice was significantly reduced at 4500 ppm. A significant decrease in the absolute and relative weight of the spleen was observed at 80 ppm. The relative weights of the liver and kidney were significantly higher at 600 ppm. At 4500 ppm, a significantly decreased absolute weight of the adrenal gland, decreased absolute and relative weights of the thymus and spleen, and increased relative weights of the brain, liver, and kidney were noted in males.

Table 9 also presents the organ weights of female F2 weanlings. A significant decrease in the body weight at sacrifice was found at 4500 ppm. The relative weight of the thymus was significantly lower at 80 ppm. Significantly increased relative weights of the liver and kidney, and reduced absolute and relative weights of the uterus were found at 600 ppm. At 4500 ppm, significantly decreased absolute weights of the brain and spleen, and absolute and relative weights of the thymus and uterus, and increased relative weights of the brain, liver and kidney were noted in females.

3.10. Hematological and blood biochemical parameters (F0 and F1 adults)

A significantly higher percent of lymphocytes was observed in male F0 adults at 4500 ppm and in female F1 adults at 600 ppm. In female F0 and male F1 adults, no significant difference was noted in the WBC or differential leukocyte count between the control and DCBS-treated groups. There were no significant changes in biochemistry parameters such as total protein, albumin and globulin in male and female F0 and F1 adult rats (data not shown).

Table 6
Organ weight of male and female F1 weanlings

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F1 weanlings examined	22	24	24	24
Body weight (g)	92.2 ± 8.0[a]	88.9 ± 6.2	88.1 ± 9.1	78.2 ± 7.2**
Brain (g)	1.69 ± 0.07[c]	1.69 ± 0.05	1.70 ± 0.08	1.63 ± 0.07**
	1.84 ± 0.13[d]	1.91 ± 0.13	1.94 ± 0.15	2.09 ± 0.17**
Thymus (mg)	359 ± 68	350 ± 53	365 ± 52	278 ± 37**
	388 ± 52[d]	393 ± 50	416 ± 61	357 ± 48
Liver (g)	4.16 ± 0.49	4.09 ± 0.37	4.09 ± 0.44	3.83 ± 0.51*
	4.51 ± 0.26[d]	4.60 ± 0.25	4.64 ± 0.15*	4.89 ± 0.28**
Kidney[b] (g)	1.023 ± 0.111	1.040 ± 0.079	1.003 ± 0.135	0.894 ± 0.069**
	1.110 ± 0.073[d]	1.171 ± 0.064*	1.137 ± 0.079	1.146 ± 0.073
Spleen (mg)	394 ± 68	352 ± 68	356 ± 61	278 ± 41**
	425 ± 52[d]	395 ± 63	405 ± 59	357 ± 48**
Adrenal[b] (mg)	25.5 ± 2.6	25.5 ± 3.3	25.0 ± 3.3	22.4 ± 2.9**
	27.8 ± 2.9[d]	28.7 ± 3.0	28.4 ± 3.1	28.8 ± 4.0
Testis[b] (mg)	561 ± 77	542 ± 64	541 ± 88	529 ± 88
	608 ± 61[d]	610 ± 55	612 ± 58	677 ± 95**
Epididymis[b] (mg)	78.6 ± 9.4	77.3 ± 9.5	75.9 ± 11.6	71.3 ± 10.2*
	85.5 ± 9.8[d]	86.9 ± 8.4	86.4 ± 11.3	91.3 ± 11.7
Ventral prostate (mg)	49.2 ± 9.8	47.5 ± 7.8	43.7 ± 10.1	42.3 ± 8.9*
	53.3 ± 9.1[d]	53.4 ± 7.8	49.6 ± 10.7	54.0 ± 9.5
No. of female F1 weanlings examined	22	24	24	24
Body weight (g)	85.9 ± 7.8	82.5 ± 6.4	82.8 ± 7.2	74.3 ± 6.7**
Brain (g)	1.62 ± 0.07	1.65 ± 0.05	1.64 ± 0.05	1.57 ± 0.05**
	1.90 ± 0.14[d]	2.01 ± 0.17	2.00 ± 0.16	2.13 ± 0.16**
Thymus (mg)	361 ± 77	327 ± 49	350 ± 60	281 ± 43**
	418 ± 65[d]	398 ± 60	423 ± 63	379 ± 56
Liver (g)	3.72 ± 0.44	3.52 ± 0.35	3.65 ± 0.40	3.43 ± 0.40*
	4.33 ± 0.34[d]	4.27 ± 0.22	4.41 ± 0.29	4.62 ± 0.31**
Kidney[b] (g)	0.954 ± 0.108	0.967 ± 0.081	0.940 ± 0.114	0.850 ± 0.082**
	1.110 ± 0.068[d]	1.173 ± 0.055**	1.133 ± 0.065	1.148 ± 0.088
Spleen (mg)	338 ± 58	323 ± 47	316 ± 53	249 ± 32**
	392 ± 43[d]	392 ± 54	382 ± 55	337 ± 49**
Adrenal[b] (mg)	23.8 ± 2.6	24.5 ± 2.7	23.1 ± 2.9	21.5 ± 2.4*
	27.8 ± 2.8[d]	29.8 ± 3.6	27.9 ± 2.6	29.1 ± 3.6
Ovary[b] (mg)	23.2 ± 3.3	22.2 ± 3.4	20.5 ± 3.2*	20.3 ± 3.2*
	27.1 ± 3.3[d]	27.0 ± 4.0	24.8 ± 4.3	27.5 ± 4.7
Uterus (mg)	58.2 ± 14.5	55.8 ± 7.6	62.1 ± 12.3	48.4 ± 11.8*
	67.9 ± 15.8[d]	67.9 ± 9.9	75.2 ± 14.1	65.0 ± 14.1

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] Relative organ weight = organ weight (g or mg)/100 g body weight.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

3.11. Serum hormone levels (F0 and F1 adults)

No significant changes in any serum hormone levels of male and female F0 adults were noted between the control and DCBS-treated groups (data not shown).

Serum hormone levels of male and female F1 adult rats are shown in Fig. 4. Although significantly higher levels of testosterone at 80 ppm and LH at 600 ppm were observed in F1 males, no significant changes were noted in any hormone levels in F1 males at 4500 ppm. There were no significant changes in any

Table 7
Organ weight of male F1 adults

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F1 adults examined	24	24	24	24
Body weight (g)	630.7 ± 74.7[a]	605.1 ± 47.7	614.2 ± 52.5	622.6 ± 51.8
Brain (g)	2.26 ± 0.10[c]	2.29 ± 0.06	2.26 ± 0.06	2.21 ± 0.09*
	0.363 ± 0.038[d]	0.380 ± 0.028	0.370 ± 0.030	0.356 ± 0.027
Pituitary gland (mg)	13.6 ± 1.4	13.9 ± 1.3	13.9 ± 1.1	14.0 ± 1.6
	2.17 ± 0.23[d]	2.30 ± 0.25	2.27 ± 0.17	2.26 ± 0.26
Thyroid[b] (mg)	24.9 ± 4.9	23.3 ± 4.7	23.8 ± 4.5	24.6 ± 4.9
	3.95 ± 0.66[d]	3.86 ± 0.78	3.88 ± 0.69	3.95 ± 0.67
Thymus (mg)	346 ± 116	269 ± 54*	331 ± 83	316 ± 62
	54.8 ± 17.0[d]	44.5 ± 8.9*	53.9 ± 12.7	50.9 ± 9.8
Liver (g)	20.80 ± 3.73	19.69 ± 2.32	21.19 ± 2.06	22.82 ± 3.37*
	3.28 ± 0.29 [d]	3.25 ± 0.19	3.46 ± 0.28	3.65 ± 0.28**
Kidney[b] (g)	3.70 ± 0.52	3.66 ± 0.23	3.69 ± 0.36	3.91 ± 0.43
	0.586 ± 0.041[d]	0.606 ± 0.042	0.602 ± 0.047	0.629 ± 0.044**
Spleen (mg)	909 ± 129	845 ± 141	847 ± 124	869 ± 162
	145 ± 16[d]	139 ± 18	138 ± 17	139 ± 17
Adrenal[b] (mg)	60.5 ± 9.8	60.3 ± 7.1	61.8 ± 7.2	61.3 ± 13.1
	9.6 ± 1.5[d]	10.0 ± 1.0	10.1 ± 1.3	9.8 ± 2.0
Testis[b] (g)	3.60 ± 0.35	3.61 ± 0.27	3.60 ± 0.27	3.78 ± 0.32
	0.575 ± 0.062[d]	0.601 ± 0.073	0.589 ± 0.066	0.610 ± 0.062
Epididymis[b] (mg)	1348 ± 138	1342 ± 67	1327 ± 111	1346 ± 118
	215 ± 24[d]	223 ± 21	217 ± 22	217 ± 19
Seminal vesicle (g)	2.30 ± 0.23	2.19 ± 0.28	2.21 ± 0.22	2.07 ± 0.26**
	0.368 ± 0.047[d]	0.364 ± 0.054	0.362 ± 0.039	0.333 ± 0.045*
Ventral prostate (mg)	838 ± 174	812 ± 181	822 ± 190	784 ± 168
	133 ± 24[d]	134 ± 28	134 ± 29	127 ± 31

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] Relative organ weight = organ weight (g or mg)/100 g body weight.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

serum hormone levels of female F1 adults between the control and DCBS-treated groups.

3.12. Sperm parameters (F0 and F1 adults)

Table 10 shows the sperm parameters in F0 and F1 adult males. No significant changes in sperm counts, percentage of motile sperm and progressively motile sperm, swimming speed and pattern, or percentage of morphologically abnormal sperm were noted in F0 adults between the control and DCBS-treated groups. A significant decrease in the mean lateral head displacement was found at 4500 ppm in F1 males.

4. Discussion

A two-generation reproductive toxicity study was performed to further evaluate the potential effects of DCBS on reproduction and development in rats.

The deaths and clinical signs observed in the present study are not thought to be attributable to the administration of DCBS, because the incidences of deaths and clinical signs were very low and inconsistent across generations, and these occurrences are not uncommon in toxicological studies.

The decreased food consumption in F0 males and females at 4500 ppm was accompanied by decreases in the body weight and body weight gain. However, lowered food consumption in F1 males at 80, 600 and 4500 ppm was occasional, inconsistent, and unaccompanied by changes in body weight or body weight gain. It seems likely that DCBS adversely affects the body weight and food consumption in F0 rats at 4500 ppm, but not in F1 rats.

Although a few F0 and F1 adults showed reproductive difficulties, necropsy and the histopathology of reproductive organs revealed no evidence of reproductive failure in these rats. Two F1 females showing abnormal estrous cycles remained in diestrus for 10–11 days, suggesting they were pseudopregnant. No significant changes in reproductive indices were noted in any

Table 8
Organ weight of female F1 adults

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of female F1 adults examined	22	22	21	23
Body weight (g)	331.9 ± 32.5[a]	331.2 ± 28.5	331.3 ± 23.1	330.2 ± 30.8
Brain (g)	2.08 ± 0.08[c]	2.17 ± 0.08**	2.15 ± 0.08*	2.08 ± 0.08
	0.632 ± 0.056[d]	0.658 ± 0.056	0.651 ± 0.043	0.633 ± 0.060
Pituitary gland (mg)	15.9 ± 2.0	16.1 ± 2.4	15.8 ± 1.8	16.1 ± 1.9
	4.83 ± 0.73[d]	4.90 ± 0.79	4.78 ± 0.52	4.89 ± 0.66
Thyroid[b] (mg)	19.0 ± 3.9	18.2 ± 2.7	17.7 ± 3.5	19.4 ± 4.1
	5.72 ± 0.98[d]	5.51 ± 0.70	5.35 ± 1.08	5.89 ± 1.15
Thymus (mg)	251 ± 69	212 ± 47	261 ± 65	211 ± 63
	75.3 ± 18.4[d]	64.1 ± 14.2	79.2 ± 20.2	64.0 ± 18.7
Liver (g)	14.55 ± 1.66	14.18 ± 2.14	14.32 ± 1.49	15.83 ± 2.11
	4.39 ± 0.28[d]	4.28 ± 0.49	4.33 ± 0.41	4.81 ± 0.59**
Kidney[b] (g)	2.37 ± 0.30	2.39 ± 0.22	2.40 ± 0.21	2.53 ± 0.26
	0.713 ± 0.046[d]	0.723 ± 0.040	0.726 ± 0.063	0.771 ± 0.080**
Spleen (mg)	632 ± 73	599 ± 63	609 ± 80	639 ± 115
	191 ± 18[d]	181 ± 15	184 ± 19	194 ± 37
Adrenal[b] (mg)	70.0 ± 9.7	73.5 ± 10.9	73.4 ± 9.3	77.5 ± 8.9*
	21.2 ± 3.2[d]	22.2 ± 3.1	22.2 ± 3.0	23.6 ± 3.2*
Ovary[b] (mg)	110.6 ± 13.0	109.1 ± 16.3	108.5 ± 12.5	108.2 ± 13.4
	33.4 ± 2.9[d]	33.0 ± 4.5	32.8 ± 3.2	32.8 ± 3.3
Uterus (mg)	927 ± 191	928 ± 128	976 ± 185	949 ± 192
	280 ± 54[d]	283 ± 48	295 ± 52	288 ± 52

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] Relative organ weight = organ weight (g or mg)/100 g body weight.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

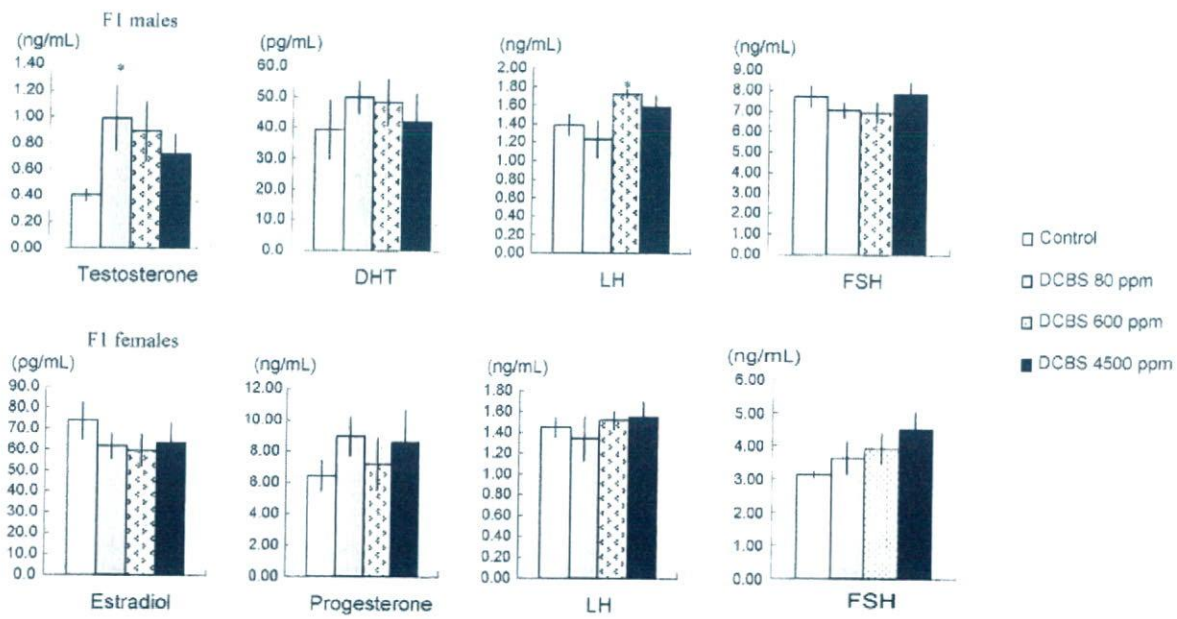

Fig. 4. Serum hormone levels in F1 males and females. The actual measurement of DHT was below the lower limit of quantification (<25.0 pg/mL) in one F1 male each in the control and 4500 ppm groups. The actual measurement of LH was below the lower limit of quantification (<0.80 ng/mL) in one F1 male and one F1 female in the 80 ppm group. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

Table 9
Organ weight of male and female F2 weanlings

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F2 weanlings examined	22	22	21	23
Body weight (g)	90.8 ± 8.7[a]	91.4 ± 13.1	89.1 ± 6.5	80.0 ± 7.8**
Brain (g)	1.67 ± 0.08[c]	1.69 ± 0.09	1.70 ± 0.08	1.63 ± 0.09
	1.85 ± 0.14[d]	1.87 ± 0.22	1.92 ± 0.13	2.05 ± 0.18**
Thymus (mg)	355 ± 56	325 ± 64	361 ± 47	283 ± 52**
	392 ± 58[d]	355 ± 47	406 ± 48	354 ± 55*
Liver (g)	4.08 ± 0.47	4.12 ± 0.66	4.21 ± 0.44	3.74 ± 0.54
	4.49 ± 0.23[d]	4.50 ± 0.26	4.72 ± 0.27**	4.66 ± 0.42**
Kidney[b] (g)	1.006 ± 0.102	1.009 ± 0.147	1.022 ± 0.089	0.923 ± 0.133
	1.109 ± 0.037[d]	1.105 ± 0.066	1.146 ± 0.049*	1.152 ± 0.117*
Spleen (mg)	383 ± 61	350 ± 83*	356 ± 46	286 ± 52**
	422 ± 49[d]	381 ± 53*	400 ± 50	357 ± 54**
Adrenal[b] (mg)	25.5 ± 2.8	24.1 ± 3.4	24.2 ± 3.8	22.7 ± 3.4*
	28.1 ± 2.8[d]	26.5 ± 2.6	27.2 ± 4.3	28.5 ± 4.2
Testis[b] (mg)	548 ± 106	516 ± 103	528 ± 82	525 ± 98
	602 ± 89[d]	563 ± 69	590 ± 65	653 ± 91
Epididymis[b] (mg)	79.5 ± 12.5	72.9 ± 12.3	72.7 ± 10.0	71.3 ± 11.2
	87.6 ± 10.6[d]	80.4 ± 12.6	81.7 ± 10.6	89.0 ± 10.0
Ventral prostate (mg)	50.9 ± 16.6	44.6 ± 10.4	47.0 ± 10.3	42.6 ± 12.2
	55.6 ± 15.0[d]	48.9 ± 9.9	52.7 ± 10.1	52.9 ± 13.1
No. of female F2 weanlings examined	22	22	20	23
Body weight (g)	83.6 ± 9.5	87.2 ± 10.8	82.4 ± 6.5	74.6 ± 7.9**
Brain (g)	1.62 ± 0.08	1.66 ± 0.07	1.66 ± 0.05	1.57 ± 0.07*
	1.96 ± 0.20[d]	1.92 ± 0.19	2.03 ± 0.16	2.11 ± 0.18*
Thymus (mg)	364 ± 50	326 ± 66	348 ± 68	283 ± 56**
	439 ± 63[d]	373 ± 56**	424 ± 80	379 ± 63**
Liver (g)	3.71 ± 0.47	3.87 ± 0.50	3.80 ± 0.37	3.57 ± 0.46
	4.44 ± 0.18[d]	4.44 ± 0.23	4.61 ± 0.19*	4.78 ± 0.26**
Kidney[b] (g)	0.915 ± 0.093	0.983 ± 0.137	0.960 ± 0.111	0.885 ± 0.101
	1.096 ± 0.046[d]	1.129 ± 0.085	1.164 ± 0.083**	1.187 ± 0.061**
Spleen (mg)	340 ± 63	331 ± 55	320 ± 46	274 ± 40**
	407 ± 58[d]	380 ± 46	389 ± 56	370 ± 52
Adrenal[b] (mg)	23.6 ± 2.9	23.3 ± 4.0	22.2 ± 3.3	21.6 ± 3.0
	28.4 ± 3.3[d]	26.7 ± 3.3	27.0 ± 4.0	29.0 ± 3.9
Ovary[b] (mg)	22.0 ± 3.9	22.5 ± 2.8	20.9 ± 3.1	21.4 ± 2.9
	26.6 ± 5.2[d]	26.0 ± 3.0	25.5 ± 4.4	29.0 ± 4.3
Uterus (mg)	61.8 ± 18.9	58.1 ± 11.9	50.0 ± 10.0*	46.6 ± 12.9**
	73.3 ± 17.2[d]	67.0 ± 13.5	60.7 ± 11.5*	62.3 ± 15.0*

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] Relative organ weight = organ weight (g or mg)/100 g body weight.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

generation even at the highest dose of 4500 ppm. Our previous screening test revealed that DCBS given by gavage to rats from day 14 before mating to day 3 of lactation caused significant decreases in the gestation index, numbers of corpora lutea, implantations, pups born and pups born alive, live birth index, and viability index at 400 mg/kg bw per day [10]. This dose also caused severe maternal toxicity and a total loss of pups until PND 4. No maternal or reproductive/developmental toxicity was detected at 100 mg/kg bw per day and below in our previous study. In the present feeding study, the mean daily intakes of DCBS were 416 and 417 mg/kg bw per day for the highest dose in F0 and F1 females, respectively. Consideration of these

Table 10
Sperm parameters in F0 and F1 males

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 males				
No. of animals	24	23	24	24
No. of testicular sperm ($\times 10^6$)				
Per testis	184.1 ± 29.3[a]	187.7 ± 28.3	184.2 ± 32.7	180.8 ± 35.4
Per g testis	111.4 ± 13.2	110.7 ± 15.7	110.6 ± 17.1	106.1 ± 18.8
No. of epididymal sperm ($\times 10^6$)				
Per cauda	268.5 ± 47.6	276.2 ± 40.3	269.9 ± 56.8	263.7 ± 62.8
Per g cauda	856.4 ± 94.4	838.9 ± 99.4	850.3 ± 122.1	844.2 ± 191.3
Percent motile	88.1 ± 9.3	92.6 ± 8.2	93.2 ± 5.9	89.4 ± 10.2
Percent progressive	70.9 ± 17.4	77.3 ± 15.3	77.4 ± 12.1	70.5 ± 22.2
Mean path velocity (μm/s)	159.6 ± 20.8	159.8 ± 19.2	162.7 ± 22.0	156.8 ± 25.3
Straight line average velocity (μm/s)	112.1 ± 22.5	114.1 ± 20.0	116.1 ± 19.3	110.5 ± 29.2
Mean curvilinear velocity (μm/s)	365.7 ± 53.4	370.1 ± 42.5	372.3 ± 49.8	358.4 ± 56.3
Mean lateral head displacement (μm)	20.1 ± 1.1	19.9 ± 1.1	20.0 ± 1.3	19.9 ± 1.0
Mean beat cross frequency (Hz)	27.9 ± 1.5	27.4 ± 1.5	27.6 ± 2.2	28.3 ± 2.3
Mean straightness (%)[b]	69.3 ± 6.6	70.7 ± 5.7	71.0 ± 4.3	69.5 ± 8.6
Mean linearity (%)[c]	30.4 ± 2.8	30.7 ± 3.0	31.3 ± 2.5	30.6 ± 4.0
Total abnormal sperm ratio (%)	1.1 ± 0.6	1.2 ± 0.8	2.4 ± 3.5	2.0 ± 2.4
Tailless sperm (%)	1.0 ± 0.6	1.2 ± 0.8	2.2 ± 3.5	1.8 ± 2.0
F1 males				
No. of animals	24	24	24	24
No. of testicular sperm ($\times 10^6$)				
Per testis	194.5 ± 23.0[a]	181.1 ± 21.3	186.3 ± 22.5	201.0 ± 33.3
Per g testis	115.3 ± 9.5	108.4 ± 14.3	111.1 ± 11.3	113.6 ± 15.0
No. of epididymal sperm ($\times 10^6$)				
Per cauda	273.6 ± 40.0	254.0 ± 40.4	256.2 ± 46.0	250.3 ± 55.4
Per g cauda	849.9 ± 69.4	821.5 ± 106.8	827.2 ± 93.3	807.0 ± 127.5
Percent motile	92.3 ± 5.0	92.9 ± 4.0	93.3 ± 5.6	93.0 ± 7.4
Percent progressive	81.8 ± 8.1	81.8 ± 4.9	83.9 ± 6.4	82.7 ± 8.2
Mean path velocity (μm/s)	175.2 ± 9.8	171.7 ± 11.2	172.4 ± 11.4	171.3 ± 13.9
Straight line average velocity (μm/s)	126.9 ± 10.2	123.9 ± 10.3	126.0 ± 10.5	125.7 ± 12.6
Mean curvilinear velocity (μm/s)	399.5 ± 19.8	391.5 ± 28.6	395.1 ± 28.6	393.6 ± 29.8
Mean lateral head displacement (μm)	21.3 ± 0.9	20.9 ± 0.8	20.8 ± 0.8	20.5 ± 1.0*
Mean beat cross frequency (Hz)	26.4 ± 1.6	26.8 ± 1.4	26.1 ± 1.6	27.0 ± 1.8
Mean straightness (%)[b]	72.5 ± 3.3	72.1 ± 2.7	73.3 ± 2.9	73.5 ± 2.8
Mean linearity (%)[c]	32.0 ± 2.1	31.9 ± 2.0	32.1 ± 1.8	32.2 ± 1.5
Total abnormal sperm ratio (%)	1.4 ± 1.3	1.1 ± 0.8	1.2 ± 1.7	1.6 ± 1.9
Tailless sperm (%)	1.3 ± 1.2	0.9 ± 0.8	1.0 ± 1.6	1.5 ± 1.8

[a] Values are given as the mean ± S.D.
[b] Mean straightness (%) = straight line average velocity/mean path velocity × 100.
[c] Mean linearity (%) = straight line average velocity/mean curvilinear velocity × 100.
* Significantly different from the control, $p < 0.05$.

findings suggests that the highest dose of DCBS in the present study may be very close to the dose that induces severe maternal and reproductive toxicity. However, the possibility remains that the difference in the degree of toxicity may be due to differences in administration method. There are some examples showing that gavage and feed administration result with differences in the toxicokinetics of chemicals [20,21]. Further studies are needed to clarify the relationship between maternal and reproductive/developmental toxicity.

Regarding developmental parameters, lowered body weights of male and female pre-weaning F1 and F2 pups were noted at 4500 ppm. These findings indicate that the dose level of 4500 ppm used in this study was potent enough to have adverse effects on the growth of pups. It is noted that there are strong correlations between developmental landmark parameters and pup body weight data, and that pup body weight data is consistently a more sensitive indicator of the developmental status of the offspring [22,23]. Although delayed completion of incisor eruption was noted in male and female F1 pups at 80 ppm and in male and female F2 pups at 80 and 4500 ppm, the delayed completion of incisor eruption was not dose-dependent and the difference from the control value was very slight. Therefore, it is unlikely that the delay of incisor eruption observed in the present study was compound-related or toxicologically significant. There were

no significant changes in indices of pre-weaning functional development in the DCBS-treated groups. The AGD is also a developmental landmark for the differentiation of the external genitalia and is commonly used as a hormonally sensitive parameter of sexual differentiation in rodents [24]. The AGD per cube root of body weight ratio provides a more appropriate adjustment when it is necessary to normalize AGD to body weight [17]. No changes were observed in the AGD per cube root of body weight ratio at any doses of DCBS in any generation. The data on the AGD indicate a lack of effect of DCBS on AGD. These findings on pre-weaning developmental parameters suggest that DCBS adversely affects the growth of offspring, but not the pre-weaning landmarks of development or reflex ontogeny. An increase in the frequency of fetuses with internal hydrocephalus was reported in rats given *N*-cyclohexyl-2-benzothiazolesulfenamide, a structurally similar compound, during organogenesis in rats [25]. However, no significantly increased incidence of pups with anomalies was detected even at the highest dose in the present and previous studies of DCBS [10,15]. Regarding post-weaning landmarks of development, delays of preputial separation at 4500 ppm and vaginal opening at 600 and 4500 ppm were observed in the present study. Although the body weight at the age of preputial separation was not different between the control and DCBS-treated groups, a higher body weight at the age of vaginal opening was found at 600 and 4500 ppm in females. Preputial separation and vaginal opening indicate the onset of sexual maturity, and the body weight is correlated with the occurrence of these events [23]. Ashby and Lefevre [26] described that delays in preputial separation can only be interpreted with confidence when they are not accompanied by losses in body weight, or when the expected delay in preputial separation due to a loss of body weight has been exceeded. They also noted that measurement of delays in preputial separation may be of value in cases of large delays, but delays of 1–2 days are difficult to interpret with confidence [26]. In the present study, the delay of preputial separation at 4500 ppm was slight (1.5 days) and was not accompanied by a change in body weight, and the age of preputial separation was within the range of the background control data (40.3–42.8 days) for the last seven years in the laboratory performed current study. It is likely that the delay in preputial separation at 4500 ppm is related to general delays in development. In female rats, the age at vaginal opening is the most commonly measured marker of puberty, and vaginal opening is an estrogen-dependent event that results from an increase in the blood estradiol levels [27]. Although the delay of female vaginal opening at 600 and 4500 ppm was slight (1.5–1.6 days), the age of vaginal opening was over the range of the background control data (29.6–31.0 days) for the last seven years in the laboratory performed present study. In the present study at 600 and 4500 ppm, the heavier body weight was noted at the completion of vaginal opening. Therefore, the possibility that the delay in vaginal opening may have toxicological meaning is not completely ruled out. Other hormone-dependent events including estrous cyclicity and AGD, as well as serum hormone levels at the scheduled terminal necropsy were not changed in the DCBS-treated groups. Moreover, DCBS did not affect the reproductive performance. However, decreased weight of the uterus was found in F1 weanlings at 4500 ppm and F2 weanlings at 600 and 4500 ppm. It has been noted that variations in the weights of the reproductive organs, which are strongly dependent on endocrine status, can be considered a key parameter in the identification of endocrine effects [28–30]. These findings suggest that DCBS may have endocrine effects. Further studies are needed to clarify the effects of DCBS on endocrine endpoints.

Regarding the behavioral tests, the only significant change in the T-maze test was observed in females on day 2 of the test. Longer elapsed times at 600 and 4500 ppm and more errors at 4500 ppm were detected in females. There are behavioral functions not classically hormone-mediated and expressed by both sexes such as learning capacities, exploration activity, novelty seeking and anxiety levels that show both qualitative and quantitative differences in the two sexes [30]. The reduced activity, as well as the other effects on neuromuscular function, could be at least partially the result of lower body weight [31] and it has been found that light body weight caused worse performance in a learning task [32]. In the present study, the spontaneous activity, swimming ability in the straight channel and body weight at the time of the T-maze test was not different in F1 females between the control and DCBS-treated groups. Thus, it seems likely that DCBS may have transiently affected learning ability in the T-maze at the highest dose administered.

The changes in weight of the organs, such as the brain, thymus, kidney, and spleen that were observed at 80 and/or 600 ppm are not thought to be due to administration of DCBS, because changes occurred sporadically and not in a dose-dependent manner. The changes in the weights of the adrenal, thyroid, and male and female reproductive organs, except for the uterus, at 600 and/or 4500 ppm seem unlikely to be attributable to administration of DCBS because of inconsistent changes across ages, sexes and generations. No consistent DCBS-related effects on serum hormone levels or sperm parameters were also detected across generations. Decreased absolute weights and/or increased relative weights of the liver except for in female F0 adults, the spleen in F0 adults, and the brain and kidney in F0 and F1 adults and F1 and F2 weanlings at 4500 ppm seem to be due to secondarily lowered body weight, but not due to the direct effects of DCBS on the organs. Decreased absolute and/or relative weights of the thymus and spleen in the weanlings are supported by the results of our previous study in which atrophy of the thymus and spleen was observed at 400 mg/kg bw per day [10]. These findings may suggest that one of target systems of DCBS toxicity is the immune system in weanlings. In the present study, however, no DCBS-related histopathological changes were detected. The discrepancy in histopathological findings between the previous and present studies could be explained by a difference in the toxicokinetics of chemicals due to differences in administration method. No DCBS-related findings were found in the hematological and blood biochemical examinations. In general, the effects of DCBS on organ weights were more pronounced in weanlings than adults. These phenomena suggest that DCBS may be more toxic before weaning than after weaning, and this possibility is supported by the lowered body weight of pups during the pre-weaning period, but not post-weaning.

Table 11
Summary of relevant findings in rat two-generation reproductive toxicity study of DCBS (80, 600 and 4500 ppm)

	F0						F1						F2					
	Male			Female			Male			Female			Male			Female		
	80	600	4500	80	600	4500	80	600	4500	80	600	4500	80	600	4500	80	600	4500
Lowered body weight			+			+			+			+			+			+
Decreased food consumption			+			+												
Delayed vaginal opening											+	+						
Worse performance in water T-maze											+	+						
Reduced spleen weight									+			+			+			+
Reduced thymus weight									+			+			+			+
Reduced uterine weight												+					+	+

In conclusion, the results of the two-generation reproductive toxicity study described here provide a more comprehensive toxicity profile of DCBS than has been previously reported. Relevant findings obtained from the present rat two-generation reproductive toxicity study of DCBS are summarized in Table 11. The NOAEL in the present study is considered to be 80 ppm (5.2 mg/kg bw per day) in rats.

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Evaluation of developmental neurotoxicity of polysorbate 80 in rats

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Abstract

The developmental neurotoxicity of polysorbate 80 (PS80) was evaluated in rats. Crl:CD(SD) rats were given drinking water containing PS80 at 0, 0.018, 0.13, 1.0, or 7.5% (0, 0.035, 0.245, 1.864, or 16.783 ml/kg bw/day) on day 0 of pregnancy through day 21 after delivery. Pregnant rats were allowed to deliver spontaneously. Potential adverse effects of pre- and post-natal exposure on the development and function of the nervous system in offspring of rats given PS80 were examined. Maternal body weight was lowered at 7.5%. Number of pups born was lowered at 7.5%. There were no compound-related effects on locomotor activity of offspring on postnatal days (PNDs) 14–15, 17–18, 20–21 and 33–37. No compound-related changes were found in developmental landmarks, sexual maturation, or reflex responses. Although decreased rate of avoidance responses was noted on PNDs 23–27 in male and female offspring at 7.5%, no compound-related changes were found in performance in the conditioned avoidance response on PNDs 60–67. Histopathological examinations of the brain revealed no toxicological changes. Lowered body weight was observed in male and female offspring at 7.5%. The NOAEL in this study was considered to be 1.0% (1.864 ml/mg/kg bw/day).

Keywords: Polysorbate 80; Tween 80; Developmental neurotoxicity; Behavior; Developmental landmarks; Rat

1. Introduction

Polysorbate 80 (PS80, CAS No. 9005-65-6, polyoxyethylene (20) sorbitan monooleate, commercially also known as Tween® 80) is a mixture of polyoxyethylene ethers of mixed partial oleic acid esters of sorbitol anhydrides and related compounds [1]. PS80 is very soluble in water and soluble in ethanol. PS80 is widely used in biochemical applications including, solubilizing proteins, isolating nuclei from cells in cell culture, growing tubercule bacilli, and emulsifying and dispersing substances in medicinal and food products [2]. PS80 is often used in foods as an emulsifier in ice cream, frozen custard, ice milk, fruit sherbet, and nonstandardized frozen desserts. PS80 is also used in yeast-defoamer formulations and as a solubilizing and depersing agent in pickles and pickle products [1]. Exposure of the general population to PS80 is mainly through its use as a food additive.

Several reports on neurobehavioral toxicity of PS80 are available. Varma et al. [3] reported that PS80 caused a decreased locomotor activity and hyperthermia at 2 ml/kg, and exhibition of paralytic activity at 10 ml/kg after oral administration, and decreased locomotor activity, depression and potentiation of the penobarbitone sleeping time at 2 ml/kg after intraperitoneal administration in mice. They concluded that intraperitoneal doses generally showed more pronounced effects than oral doses, and PS80 did not show any neuropharmacological effects in a dose not more than 1 ml/kg when given either intraperitoneally or orally [3]. PS80 also caused behavioral and neurochemical changes in cats after intraperitoneal administration [4,5]. Intraperitoneal injection of 0.1% saline solution of PS80 in a volume of 3 ml/kg three times every 12 h decreased the carbachol-induced growing response and increased the content of 5-hydroxyindoleacetic acid in the hypothalamus in cats [4]. As for the developmental neurotoxicity of PS80, Brubaker et al. [6] reported that locomotor activity was enhanced in pre-weaning male offspring of rats received drinking water containing PS80 at 1.25 ml/l (0.125%) during the pre-mating, mating, pregnancy and lactation periods. However, their study did not provide enough information on all aspects of developmental neurotoxicity due to the use of one dose group and the

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selection of endpoints. Only pre-weaning locomotor activity in male offspring was determined, and no other parameters were evaluated in their study. The present study was therefore conducted to further evaluate the developmental neurotoxicity of PS80, including locomotor activity, in rats using a study design similar to the OECD Draft Proposal for New Guideline 426, Developmental Neurotoxicity Study [7].

2. Materials and methods

This study was performed in accordance with the principles for Good Laboratory Practice [8]. This study was conducted also in compliance with the "Law of Humane Treatment and Management of Animals" [9] and "Guidance for Animal Care and Use" of Ina Research Inc. and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee of Ina Research Inc. fully accredited by AAALAC International [Accredited Unit No. 001107].

2.1. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male rats at 10 weeks of age and female rats at 9 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days, prior to the start of the experiment, and rats found to be in good health were selected for use. Vaginal smears of each female rat were recorded, and rats showing regular estrous cycles were used in the experiment. Animals were reared on a basal diet (NMF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*, and were maintained in an air-conditioned room at 21.0–25.0 °C, with a relative humidity of 40–70%, a 12-h light (7:00–19:00)/dark (19:00–7:00) cycle, and ventilation of 16 air changes/h. Virgin female rats were mated overnight with male rats. The day when sperm was detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 215–324 g, were distributed into five groups of 22 females to equalize the body weights among groups. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared on sterilized wooden chips (Sun Flake: Charles River Laboratories Japan, Inc.).

2.2. Chemical and dosing

Polysorbate 80 (PS80) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The PS80 used in this study was a technical grade (Lot no. EWP7301/code no. 162-21771, saponification value: 49.3, hydroxyl value: 70.1), and was kept in a dark and cool place. The stability of the PS80 was verified by analysis before and after the study. Rats were given PS80 in their drinking water at a concentration of 0 (control), 0.018, 0.13, 1.0, or 7.5% on day 0 of pregnancy through day 21 after delivery. The dosage levels were determined based on the results of our previous dose-finding study, in which decreased body weight gain and food and water consumption at 10.0% and higher, slight decrease in the body weight gain and food consumption at 7.5%, and no adverse effects at 5.0% and below were observed in female rats given PS80 in their drinking water for 14 days (data not shown). Dosed water preparations were formulated by mixing and dissolved PS80 into an appropriate amount of distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan) for each water concentration. Rats were given PS80 at a constant water concentration. The control rats were given only water. The stability of formulations at room temperature has been confirmed for up to 7 days. During use, formulations were maintained at room temperature for not more than 5 days, and were 100.4–108.6% of the target concentration.

2.3. Observations of dams

All pregnant rats were observed daily for clinical signs of toxicity. Maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. Female rats were checked for signs of parturition before and after noon from days 20 to 25 of pregnancy to determine the time of delivery. The day on which parturition was completed by 16:00 was designated as day 0 after delivery. The females were allowed to deliver spontaneously and nurse their pups until day 21 after delivery. Parental female rats were euthanized by exsanguination under isoflurane anesthesia on day 21 after delivery. The external surfaces of rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For each female, the number of uterine implantation sites was recorded, and the weights of the brain, liver, kidney, spleen, and adrenal were determined.

2.4. Observations of offspring

The day of birth was designated as postnatal day (PND) 0. On PND 0, total litter size and the numbers of live and dead pups were recorded, and pups were counted, sexed, and examined grossly on PND 0. All pups were observed daily for clinical signs of toxicity, and individually weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, each of the litters was randomly adjusted to eight pups comprising of four males (1m, 2m, 3m and 4m) and four females (1f, 2f, 3f and 4f). Litters of less than eight pups were not used in the experiment. All pups were observed daily for pinna unfolding beginning on PND 2, fur appearance and incisor eruption beginning on PND 8, and eye opening beginning on PND 12. Body weights of pups were recorded on the day of completion of these developmental landmarks. Pups were weaned on PND 21.

2.4.1. Functional/behavioral observations during the pre-weaning period

One male (1m) and one female (1f) pup selected from all dams in each group was evaluated for surface righting reflex on PND 5, and negative geotaxis reflex on PND 8. Locomotor activity of offspring (1m and 1f) on PNDs 14–15, 17–18, and 20–21 at 20:00, 2:00, 8:00 and 14:00 was determined in the open field. Subject rats were placed individually in a box (26 cm in length and width, and 20 cm height) in a 3 × 3 matrix, consisting of a black acrylic plate, with a camera directly overhead and were allowed to explore freely for 10 min. The distance traveled by each monitored rat was recorded with video-based tracking software (BigBrother, Actimetrics, Inc., Wilmette, IL). Locomotor activity was determined under white noise (60 dB) to attenuate external sound, and light at 166–300 lx during the diurnal period and an infrared lamp during the nocturnal period.

2.4.2. Functional/behavioral observations during the adolescent and young adult periods

All remaining male (2m, 3m and 4m) and female (2f, 3f and 4f) pups of each dam were observed daily for clinical signs of toxicity, and individually weighed on PNDs 28, 35, 42, 49, 56 and 70.

Male (2m) and female (2f) pups selected from all dams in each group were evaluated for pupillary reflex, Preyer's reflex, pain response and mid-air righting on PNDs 23–26 and 62–64, and locomotor activity was determined on PNDs 33–37 and 60–66. An open-field with a box (39 cm in length and width, and 30 cm height) in a 2 × 2 matrix was used to evaluate locomotor activity in post-weaning offspring. Other procedures for the determination of locomotor activity were the same as described above for pre-weaning pups. Offspring (2m and 2f) were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. The body weight of the respective rats was recorded on the day of preputial separation or vaginal opening.

Conditioned avoidance response was determined on PNDs 23–27 in male (3m) and female (3f) pups of half of the dams in each group, and on PNDs 60–67 in male (3m) and female (3f) pups of the other half of the dams in each group. The shuttle box (40 cm in length, 20 cm width, and 20 cm height), which consisted of transparent acrylic plastic panels, was divided into two equal compartments by a roller (40 and 55 mm in diameter for pups on PNDs 23–27 and PNDs 60–67, respectively). A rat placed in one compartment could get over the roller and cross to the other side. The grid floor of each compartment consisted of stainless steel rods spaced at 10 mm (for pups on PNDs 23–27) or 13 mm (for pups on PNDs 60–67) center to center. An electric shock could be delivered through the grid floor of the occupied compartment from a shock generator/scrambler (MU Co., Chino, Japan). A subject rat was given 2 min to adapt to the shuttle box after its introduction into one compartment. The trial began with a warning buzzer

(2000 Hz, 25 dB) as the conditioned stimulus (CS) for 5 s. A rat crossing to the opposite side of the shuttle box during the buzzer period would successfully avoid the electric shock (3 mA) that followed the buzzer. If the rat had not yet crossed to the opposite compartment of the shuttle box after the 5 s buzzer period, an electric shock was applied for 10 s, as the unconditioned stimulus. A 30 s intertrial interval preceded the next presentation of the CS. Each rat was tested for 60 min a day for three consecutive days. The rate of successfully conditioned responses for every 10 and 60 min was calculated.

One male (4m) and one female (4f) pups selected from each dam were maintained as reserve animals for replacements or additional tests.

2.4.3. Necropsy of offspring

Humane sacrifice was performed on PND 22 for pups (1m and 1f), on PND 70 for pups (2m, 3m, 2f and 3f), and on PNDs 103–126 for pups (4m and 4f) of each dam. The external surfaces of pups were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For histopathological examinations, half of pups in each group (10–11/sex/group) killed on PNDs 22 or 70 were perfused with heparinized phosphate-buffered solution and paraformaldehyde-phosphate buffered solution, and the brain, spinal cord in the thoracic and lumbar regions, and sciatic nerve were removed and stored in 10% neutral buffered formalin. Histopathological evaluations were performed on the cerebrum, cerebellum, medulla oblongata, pons, spinal cord and sciatic nerve of male and female pups in the control and highest dose groups after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin. The remaining pups (8–11/sex/group) killed on PNDs 22 or 70 were subjected to weighing of the brain, liver, kidney, spleen and adrenal.

2.5. Statistical analysis

Statistical analysis of offspring before weaning was carried out using the litter as the experimental unit. The initial body weight, body weight gain and food and water consumptions of maternal rats, numbers of implantations and pups per litter, organ weight, pup weight, day of completion of developmental landmarks, latency of reflex response, distance traveled by pups, rate of avoidance response were analyzed with Bartlett's test [10] for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test [11] to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the PS80-treated groups was compared with that of the control group with the Dunnett-type test which was used for the gestation length, delivery index, incidence of pups with malformations, viability index of pups and rate of pups that completed reflex responses to compare the mean rank of groups treated with PS80 and the control group. The fecundity index and gestation index were analyzed by chi-square test. The 0.05 level of probability was used as the criterion for significance.

3. Results

3.1. Findings in dams

No feces were found in one female on days 20–21 of pregnancy and on day 0 of lactation at 7.5%. During lactation, loose stools in 18 females on days 2–21, and scattering of offspring on days 0–1, scant or no feces on days 1–2, reddish brown soiled perianal region on days 12 and 20 and death of all offspring on day 2 in one female each were observed at 7.5%. No clinical signs of toxicity were noted at 1.0% and below (data not shown).

The body weights of maternal rats during pregnancy and lactation are shown in Fig. 1. A significantly lower body weight was observed on days 3, 10, 12–20 of pregnancy and days 0, 2–18 of lactation at 7.5%. In this group, body weight gain was also significantly decreased during pregnancy.

A significant decrease in food consumption on all measuring days during pregnancy and lactation was noted at 7.5%. No

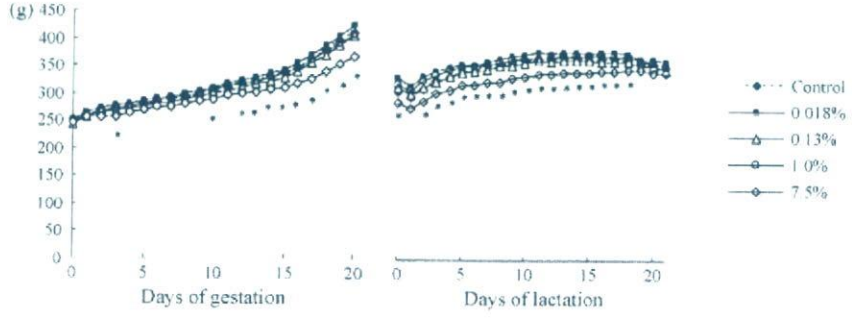

Fig. 1. Body weight of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

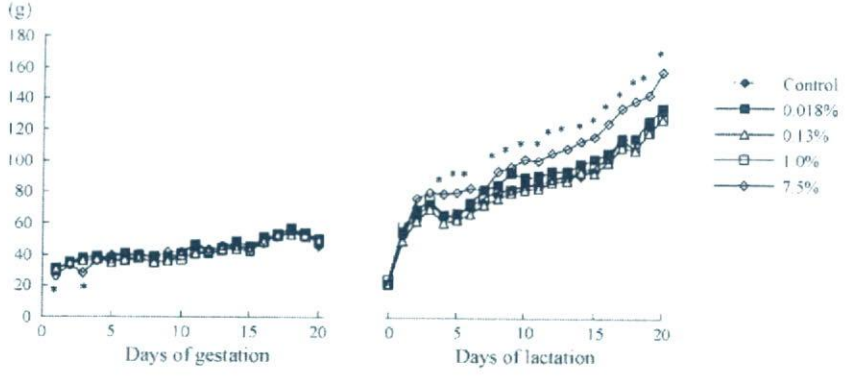

Fig. 2. Water consumption of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

significant changes in food consumption were found at 1.0% and below (data not shown).

The water consumption of maternal rats during pregnancy and lactation are shown in Fig. 2. At 7.5%, water consumption was significantly decreased on days 1 and 3 of pregnancy and increased on days 5–7 and 9–21 of lactation. No significant changes in water consumption were found at 1.0% and below. The least water consumption was observed on day 0 of lactation and the most water consumption was observed on day 21 of lactation in all groups.

The average daily intakes of PS80 during pregnancy were 0.024, 0.171, 1.314 and 10.576 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intake of PS80 during lactation were 0.046, 0.321, 2.521 and 23.908 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intakes of PS80 throughout the administration period were 0.035, 0.245, 1.864 and 16.783 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The least intake of PS80 was noted on day 0 of lactation and the most intake of PS80 was noted on day 21 of lactation in all groups. The ranges of average daily intakes of PS80 based on each day of the administration period were 0.012–0.071, 0.093–0.499, 0.837–3.765 and 5.869–36.431 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively.

At necropsy of dams, dilatation of the cecum in seven females and a significant increase in the relative weight, but not in the absolute weight, of the kidney, were observed at 7.5%. No changes in gross pathology or in absolute and relative weights were detected in any organs at 1.0% and below (data not shown).

3.2. Reproductive/developmental findings

The reproductive findings in maternal rats are presented in Table 1. One female at 7.5% showed total litter loss by day 2 of lactation, one female at 0.018 and 1.0% and two females at 0.13% were not impregnated, and one pregnant female at 1.0%, died on day 22 of pregnancy; however, no significant differences were noted in the fecundity index, gestation index and length of gestation between control and PS80-treated groups.

The developmental findings are shown in Table 2. The number of pups born was significantly reduced at 7.5%. There were no significant effects of treatment of PS80 on the numbers of implantations, pups born alive and dead pups, delivery index and sex ratio of pups and the viability index of pups before weaning. A fetus with acaudate and anal atresia was observed at 0.018%, but no fetuses with external malformations were found in other groups. Although no significant changes in the body weight of male and female pups were observed on PNDs 0, 4 and 7 in PS80-treated groups, significantly reduced body weights were noted on PNDs 14 and 21 at 7.5%. No PS80-related clinical signs of toxicity were found during the pre-weaning period.

The body weights after weaning in male and female offspring of rats given PS80 during pregnancy and lactation are shown in Table 3. At 7.5%, a significantly reduced body weight was noted on PNDs 28, 35, 42, 49 and 56 in males and on PNDs 28 and 35 in females.

One male at 1.0% died on PND 23; however, there were no compound-related clinical signs of toxicity or adverse effects on the survival rate in male and female weaned rats (data not shown).

3.3. Developmental landmarks in offspring

Physical development in male and female pups is presented in Table 4. There was no significant difference in the age of male and female pups that displayed pinna unfolding, fur appearance, incisor eruption, or eye opening. Body weight at the age of fur appearance in males and eye opening in both sexes was significantly reduced at 7.5%.

Data on sexual development in male and female pups is shown in Table 5. No significant differences in age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening were found between control and PS80-treated groups.

Examination of reflex ontogeny revealed no significant difference between control and PS80-treated groups in the latency of response, i.e., the time taken by the subject to complete reflex response, or the incidence of pups completing reflex response. All male and female pups in all groups, except for one male pup at 1.0%, showed completion of the reflex response when testing surface righting reflex on PND 5 and negative geotaxis reflex on

Table 1
Reproductive findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of females copulated	22	22	22	22	22
No. of pregnant females	22	21	20	21	22
No. of non-pregnant females	0	1	2	1	0
Fecundity index (%)[a]	100	95.5	90.9	95.5	100
No. of deaths during pregnancy	0	0	0	1	0
Gestation length (days)[b]	21.9 ± 0.4	21.8 ± 0.4	21.9 ± 0.4	21.9 ± 0.4	21.7 ± 0.5
No. of females with live born	22	21	20	20	22
Gestation index (%)[c]	100	100	100	95.2	100
No. of females with totally litter loss	0	0	0	0	1

[a] Fecundity index (%) = (no. of pregnant females/no. of females confirmed mating) × 100.
[b] Values are given as the mean ± S.D.
[c] Gestation index (%) = (no. of females with live pups born/no. of pregnant females) × 100.

Table 2
Developmental findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of litters	22	21	20	21[a]	22
No. of implantations[b]	16.1 ± 1.7	15.4 ± 2.7	15.1 ± 1.6	15.8 ± 1.4	15.1 ± 2.1
Total no. of pups born[b]	15.4 ± 1.8	14.8 ± 2.4	14.2 ± 2.1	14.6 ± 1.2	13.9 ± 1.7*
No. of pups born alive[b]	14.7 ± 1.8	14.6 ± 2.3	13.7 ± 2.0	14.5 ± 1.2	13.6 ± 2.0
No. of dead pups[b]	0.7 ± 1.2	0.1 ± 0.4	0.5 ± 0.7	0.2 ± 0.7	0.3 ± 0.8
Delivery index (%)[c]	95.8	96.0	94.1	92.9	92.8
Proportion of male pups (%)[d]	54.2	49.2	53.6	47.7	44.7
Viability index before weaning (%)					
Postnatal day 0[e]	95.5	99.1	96.6	99.0	97.6
Postnatal day 4[f]	98.6	98.2	98.5	98.7	93.8
Postnatal day 21[g]	100	100	100	100	100
Body weight of male pups before weaning (g)[b]					
Postnatal day 0	6.4 ± 0.5	6.5 ± 0.5	6.5 ± 0.5	6.5 ± 0.4	6.1 ± 0.8
Postnatal day 4	10.1 ± 1.0	10.6 ± 1.3	10.4 ± 1.3	10.4 ± 0.8	9.8 ± 1.1
Postnatal day 7	16.9 ± 1.4	17.6 ± 1.5	17.1 ± 1.9	17.4 ± 1.1	16.2 ± 1.8
Postnatal day 14	35.3 ± 1.6	36.5 ± 2.4	34.8 ± 2.5	35.6 ± 1.8	32.4 ± 3.3*
Postnatal day 21	58.0 ± 3.6	59.0 ± 4.1	57.0 ± 3.5	57.1 ± 3.0	50.4 ± 5.1*
Body weight of female pups before weaning (g)[b]					
Postnatal day 0	6.0 ± 0.5	6.2 ± 0.5	6.2 ± 0.5	6.1 ± 0.3	5.8 ± 0.7
Postnatal day 4	9.6 ± 1.0	10.2 ± 1.1	9.9 ± 1.3	9.9 ± 0.8	9.5 ± 1.0
Postnatal day 7	16.1 ± 1.4	17.0 ± 1.6	16.3 ± 1.9	16.6 ± 1.1	15.7 ± 1.8
Postnatal day 14	34.0 ± 1.9	35.2 ± 2.3	33.5 ± 2.3	34.4 ± 1.4	31.5 ± 3.1*
Postnatal day 21	55.0 ± 3.7	56.4 ± 4.0	54.5 ± 3.1	55.1 ± 2.0	49.2 ± 4.7*
External examination of pups[h]					
No. of pups (litters) examined	339 (22)	310 (21)	284 (20)	278 (19)	306 (22)
No. of pups with malformations	0	1[i]	0	0	0

[a] One female, who delivered four live pups on day 23 of pregnancy, was euthanized on day 0 after delivery, and her data were excluded.
[b] Values are expressed as the mean ± S.D.
[c] Delivery index (%) = (no. of pups born/no. of implantations) × 100.
[d] Proportion of male pups = (no. of male pups/total no. of pups) × 100.
[e] Viability index on postnatal day 0 (%) = (no. of pups born alive/total no. of pups born) × 100.
[f] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of pup born alive) × 100.
[g] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live on postnatal day 4 after cull) × 100.
[h] External examinations were performed on all pups born on postnatal day 0.
[i] One live pup had acaudate and anal atresia.
* Significantly different from the control, $p < 0.05$.

PND 8. As for the sensory function of offspring, all male and female pups in all groups completed pupillary reflex, Preyer's reflex, pain response and mid-air righting reflex when tested on PNDs 23–26 and 62–64 (data not shown).

3.4. Locomotor activity in offspring

Locomotor activity of male and female pups during the pre-weaning period is presented in Fig. 3. No significant differences in the distance traveled by male and female pups during the nocturnal period (20:00 and 2:00) and diurnal period (8:00 and 14:00) were found between control and PS80-treated groups when locomotor activity was determined on PNDs 14–15. Although a significantly higher activity was observed in male pups in the 1.0% group at 2:00 on PND 18, no significant changes in activity were noted in males and females at any other test time on PNDs 17–18. There were no significant differences between control and PS80-treated groups in locomotor activity of male and female offspring at any test time on PNDs 20–21.

After weaning, no significant differences in the distance traveled by male and female offspring were detected at any test time when activity was determined on PNDs 33–37 and PNDs 60–66 (data not shown).

3.5. Conditioned avoidance response in offspring

The rate of successfully conditioned responses for every 10 min test period on PNDs 23–27 is presented in Fig. 4. On the first day of the test, the rate of successful responses for 60 min was lower in males and females at 7.5%, and a significantly decreased rate was noted in males during the last 10 min and in females during the first and third 10 min test periods. However, there were no significant changes in the rate of successful responses in any 10 min test periods in males and females of any PS80-treated groups on the second- and third-day of the test. No significant changes in the total rate of successfully responses for 60 min were found in male and female pups in any PS80-treated groups on any test days.

Table 3
Body weight after weaning in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male offspring	63	61	60	55	60
Body weight of male offspring (g)[a]					
Postnatal day 28	101 ± 7	102 ± 8	98 ± 6	100 ± 6	92 ± 9**
Postnatal day 35	161 ± 12	163 ± 13	157 ± 10	160 ± 10	151 ± 13**
Postnatal day 42	225 ± 15	230 ± 16	221 ± 14	225 ± 14	217 ± 15**
Postnatal day 49	289 ± 20	297 ± 20	285 ± 18	289 ± 18	281 ± 17*
Postnatal day 56	346 ± 24	356 ± 24	342 ± 22	345 ± 24	336 ± 22*
Postnatal day 63	390 ± 27	400 ± 27	384 ± 26	388 ± 27	379 ± 25
Postnatal day 70	424 ± 32	436 ± 30	418 ± 30	422 ± 32	414 ± 33
No. of female offspring	63	62	58	57	63
Body weight of female offspring (g)[a]					
Postnatal day 28	90 ± 7	93 ± 6	90 ± 5	90 ± 5	85 ± 7**
Postnatal day 35	135 ± 9	138 ± 11	135 ± 9	135 ± 7	130 ± 10*
Postnatal day 42	171 ± 13	175 ± 13	172 ± 12	171 ± 10	168 ± 13
Postnatal day 49	196 ± 14	204 ± 15*	200 ± 15	199 ± 12	196 ± 15
Postnatal day 56	222 ± 17	230 ± 17*	226 ± 17	222 ± 14	221 ± 18
Postnatal day 63	239 ± 19	248 ± 19*	246 ± 22	243 ± 17	241 ± 19
Postnatal day 70	254 ± 20	264 ± 22*	262 ± 22	258 ± 18	257 ± 21

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

Table 4
Physical development in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No of litters examined	22	21	20	19	21
Age at pinna unfolding (days)[a]					
Male	2.9 ± 0.6	3.0 ± 0.5	3.0 ± 0.5	2.9 ± 0.7	3.1 ± 0.5
Female	2.8 ± 0.5	2.9 ± 0.5	3.0 ± 0.5	3.1 ± 0.6	3.1 ± 0.6
Body weight at pinna unfolding (g)[a]					
Male	8.6 ± 0.7	9.3 ± 1.3	9.0 ± 1.0	8.9 ± 0.5	8.8 ± 1.0
Female	8.1 ± 0.8	8.8 ± 1.0	8.6 ± 1.0	8.5 ± 0.5	8.4 ± 0.9
Age at fur appearance (days)[a]					
Male	9.0 ± 0.4	9.0 ± 0.4	9.0 ± 0.6	9.1 ± 0.5	9.0 ± 0.5
Female	9.1 ± 0.4	9.1 ± 0.4	9.2 ± 0.6	9.2 ± 0.5	9.1 ± 0.6
Body weight at fur appearance (g)[a]					
Male	22.2 ± 1.5	23.2 ± 1.8	21.9 ± 1.8	22.5 ± 1.6	20.9 ± 2.2*
Female	21.5 ± 1.5	22.3 ± 1.7	21.2 ± 1.8	22.0 ± 1.5	20.5 ± 2.3
Age at incisor eruption (days)[a]					
Male	10.0 ± 0.8	10.1 ± 0.5	10.1 ± 0.5	10.1 ± 0.7	10.1 ± 0.9
Female	10.0 ± 0.6	10.0 ± 0.7	10.1 ± 0.4	9.9 ± 0.6	10.0 ± 1.0
Body weight at incisor eruption (g)[a]					
Male	24.6 ± 2.2	25.7 ± 1.6	24.4 ± 2.5	25.0 ± 1.8	23.1 ± 3.2
Female	23.5 ± 1.6	24.3 ± 1.7	23.6 ± 2.5	24.0 ± 1.3	22.4 ± 3.0
Age at eye opening (days)[a]					
Male	15.3 ± 0.5	15.2 ± 0.7	15.3 ± 0.5	15.4 ± 0.7	15.3 ± 0.6
Female	15.4 ± 0.5	15.2 ± 0.5	15.2 ± 0.5	15.3 ± 0.6	15.2 ± 0.6
Body weight at eye opening (g)[a]					
Male	37.9 ± 1.6	39.4 ± 2.4	37.7 ± 2.6	38.5 ± 2.0	35.1 ± 3.1*
Female	36.9 ± 1.6	37.6 ± 2.0	36.0 ± 2.1	37.3 ± 1.7	34.1 ± 3.1*

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.