7 Results and Discussion

7.1 Effects on neonatal, juvenile and pubertal rats (Andrade *et al.*, 2006a)

Perinatal exposure to DEHP and other phthalates has been described to disrupt the androgen-dependent development of male offspring rats by reducing the testosterone production of fetal/neonatal testis (Parks *et al.*, 2000; Mylchreest *et al.*, 2002). In the present study, reduced anogenital distance and nipple retention, both sensitive indicators of prenatal androgen insufficiency, were only observed in males exposed to the highest DEHP dose (405 mg/kg/day). In addition, no changes were detected in the levels of intratesticular testosterone of newborn rats at any dose.

Examination of testes by light microscopy revealed histopathological alterations on postnatal days (PNDs) 1 and 22 in the two highest dose groups (135 and 405 mg/kg/day). The most prominent finding on PND 1 was the presence of bi- and multinucleated (enlarged) gonocytes, which frequently showed signs of degeneration. On PND 22, we observed signs of reduced germ cell differentiation in seminiferous tubules of exposed animals. In opposition to normal tubules, which displayed stratified multiple layered germ cells in various stages of differentiation, affected tubules showed only one or two layers of fairly homogeneous cells, most likely gonocytes accompanied by Sertoli cells. These histopathological alterations are in agreement with the results of previous studies with high doses of phthalates and are believed to be associated with abnormal Sertoli cell function and disruption of Sertoli-germ cells interactions (Mylchreest *et al.*, 2002; Gray *et al.*, 2000; Parks *et al.*, 2000).

Testis weight on PND 22 was significantly increased in the 5, 15, 45 and 135 mg/kg/day doses, but not in the 405 mg/kg/day group, which showed a trend towards reduction (not statistically significant). Moreover, the diameter of seminiferous tubules followed the same pattern observed for testis weight. The observed increase in testis weight is in contrast with the effects reported by studies employing higher DEHP doses, where affected animals displayed reduced testis weight and tubular atrophy (Gray *et al.*, 2000; Moore *et al.*, 2001). The reason for this biphasic response at this age is unknown. Later in life (adulthood), however, no significant changes were detected in testis weight, indicating a transient effect (Andrade *et al*., 2006c).

A significant delay in the age at preputial separation was observed in the groups exposed to 15, 45, 135 and 405 mg DEHP/kg/day. This developmental landmark is used as a marker of puberty onset in rats (US EPA, 1996). In the present

study, the observed delay in the age at preputial separation (>= 15 mg DEHP/kg/day) seems to be unrelated to changes in the androgenic status, as other androgen-dependent endpoints (nipple retention and anogenital distance) were not affected at these doses, with the exception of the highest dose group (405 mg/kg/day). Interestingly, when female littermates were evaluated for vaginal opening a significant delay was observed at the same doses causing delayed preputial separation in males (Grande *et al.*, 2006).

Overall, our results confirm previous observations that DEHP acts as an anti-androgen at high doses but also indicate that other subtle developmental effects (e.g., delayed preputial separation and increased testis weight) occur at lower doses.

7.2 Effects on brain aromatase activity (Andrade *et al*., 2006b)

It has been suggested that DEHP may interfere with estrogen metabolism through suppression of aromatase enzyme activity. This enzyme catalyzes the conversion of testosterone to estradiol and plays a critical role in sexual differentiation (masculinization) of specific brain regions (George and Ojeda, 1982; Lephart, 1996). In the present study, the effects of DEHP on brain hypothalamic pre-optic area (HPOA) aromatase activity were investigated in male and female rats on postnatal days (PNDs) 1 and 22. In newborn males (PND 1), the dose-response curve obtained was nonmonotonic and J-shaped with low dose inhibition and high dose stimulation. Inhibition was statistically significant at 0.135 and 0.405 mg DEHP/kg/day, while increased activity was observed at 15, 45 and 405 mg/kg/day. No significant changes in HPOA aromatase activity were detected in females for any dose at this age. In contrast to the results on PND 1, males were largely unaffected at weaning (PND 22), indicating that the observed effects on aromatase activity were transient. Females on PND 22, however, presented increased HPOA aromatase activity, which was significant in all doses except for 0.045 and 5 mg/kg/day. These results suggest that there are temporal differences between males and females in aromatase susceptibility to DEHP exposure. However, the exact mechanisms underlying DEHP effects on aromatase are difficult to determine, as the endogenous factors controlling the developmental expression of this enzyme in the rat are not fully understood (Lephart, 1996).

The observed changes in brain aromatase activity in newborn males were not associated with impairment of male sexual behaviour later in life (Andrade *et al*.,

2006c). Future investigations should determine whether DEHP can also induce changes in aromatase enzyme in other target organs such as the fetal testis. This could, for instance, clarify some aspects of male reproductive toxicity of phthalates, as it has been hypothesized that the imbalance between androgen and estrogen actions could be responsible for the effects of phthalates on testis development (Sharpe, 2001).

7.3 Reproductive effects on adult offspring (Andrade *et al*., 2006c)

The major effects of DEHP on adult offspring rats were impairment of testicular function and induction of reproductive tract abnormalities. A reduction in daily sperm production of 19-25% relative to control was observed in animals exposed to 15, 45, 135 and 405 mg/kg/day. When we investigated the effects of DEHP on rat reproductive performance, no alterations were observed in time to mating and fertility/pregnancy indices at any dose. This is not surprising, as reductions of up to 90% of sperm production in rats and mice were reported not to adversely affect fertility (Mesitrich, 1982; Faqi *et al.*, 1997). However, less severe effects can have dramatic consequences for human males who function nearer the threshold for the number of sperm needed to ensure reproductive competence (Zenick and Clegg, 1989). Our results on testicular morphometry and cell counts show that the observed effects in daily sperm production are not related to changes in the number of Sertoli cells or their capability to support early stage spermatocytes (as indicated by unchanged ratio of leptotene spermatocytes to Sertoli cells).

In addition to the effects on sperm production, a low incidence of reproductive tract malformations (cryptorchidism and small scrotal testes) was detected in DEHP exposed animals. Undescended (ectopic) testes were observed in three animals, which were exposed to 5, 135 and 405 mg DEHP/kg/day. This reproductive tract malformation, characteristic of the “phthalate syndrome”, is unusual among control rats and can be considered a biologically significant finding. Examination of testes by light microscopy indicated that the most prominent histopathological changes appeared in ectopic (undescended) or macroscopically altered testes. Histopathological findings ranged from reduced spermatogenesis with loss of germ cell stratification to severe atrophy of seminiferous tubules. In addition, Leydig cell hyperplasia and presence of enlarged multinucleated cells, alterations previously reported in association with phthalate exposure (Barlow and Foster, 2003; Shirota *et*

al., 2005), were observed in animals exposed to the highest DEHP dose (405 mg/kg/day) but not in control or lower dose groups.

Serum testosterone concentration was unchanged at most doses, but significantly increased at 0.045, 0.405 and 405 mg DEHP/kg/day. This effect was particularly evident in the 405 mg/kg/day group in which testosterone levels were more than two times higher in relation to control values. Interestingly, this was the only dose at which Leydig cell hyperplasia was noted in testicular sections. In spite of the increase in serum testosterone concentration at 405 mg/kg/day, the weight of the seminal vesicle plus coagulating glands (testosterone-dependent tissues) was significantly reduced at this dose. According to Gray *et al*. (2000), the testosterone insufficiency induced by phthalates during perinatal development can impair the hormonal imprinting of sex accessory organs, which cannot attain full responsiveness to androgens later in life. The observed reduction in seminal vesicle weight is in accordance with our previous results showing anti-androgenic effects (reduced anogenital distance and nipple retention) at 405 mg DEHP/kg/day (Andrade *et al*., 2006a).